

'STUDIES ON BABESIOSIS,  
WITH PARTICULAR REFERENCE TO CHRONIC PARASITAEMIAS'.

ALEXANDER JOHN TREES

B.V.M. & S. (Edin.), M.R.C.V.S.

Thesis presented for the degree of Doctor of Philosophy  
in the Faculty of Veterinary Medicine,  
University of Edinburgh, 1976.



1. Introduction	1
2. Materials and Methods	2
3. Results	3
4. Discussion	4
5. Conclusion	5
6. Acknowledgments	6
7. References	7
8. Appendix	8
9. Index	9
10. Glossary	10
11. Bibliography	11
12. Tables	12
13. Figures	13
14. Plates	14
15. Photographs	15
16. Maps	16
17. Diagrams	17
18. Schemata	18
19. Flowcharts	19
20. Tables	20
21. Figures	21
22. Plates	22
23. Photographs	23
24. Maps	24
25. Diagrams	25
26. Schemata	26
27. Flowcharts	27
28. Tables	28
29. Figures	29
30. Plates	30
31. Photographs	31
32. Maps	32
33. Diagrams	33
34. Schemata	34
35. Flowcharts	35
36. Tables	36
37. Figures	37
38. Plates	38
39. Photographs	39
40. Maps	40
41. Diagrams	41
42. Schemata	42
43. Flowcharts	43
44. Tables	44
45. Figures	45
46. Plates	46
47. Photographs	47
48. Maps	48
49. Diagrams	49
50. Schemata	50
51. Flowcharts	51
52. Tables	52
53. Figures	53
54. Plates	54
55. Photographs	55
56. Maps	56
57. Diagrams	57
58. Schemata	58
59. Flowcharts	59
60. Tables	60
61. Figures	61
62. Plates	62
63. Photographs	63
64. Maps	64
65. Diagrams	65
66. Schemata	66
67. Flowcharts	67
68. Tables	68
69. Figures	69
70. Plates	70
71. Photographs	71
72. Maps	72
73. Diagrams	73
74. Schemata	74
75. Flowcharts	75
76. Tables	76
77. Figures	77
78. Plates	78
79. Photographs	79
80. Maps	80
81. Diagrams	81
82. Schemata	82
83. Flowcharts	83
84. Tables	84
85. Figures	85
86. Plates	86
87. Photographs	87
88. Maps	88
89. Diagrams	89
90. Schemata	90
91. Flowcharts	91
92. Tables	92
93. Figures	93
94. Plates	94
95. Photographs	95
96. Maps	96
97. Diagrams	97
98. Schemata	98
99. Flowcharts	99
100. Tables	100

To the memory of my mother and father



## CONTENTS

	Page
Summary	1
Introduction and Literature Review	6
Materials and Methods	22
A. Experiments on rodents	22
(i) Animals	22
(ii) <u>Babesia</u>	23
(iii) Observations	24
(iv) Techniques	26
B. Experiments on cattle	29
(i) Animals	29
(ii) <u>Babesia</u>	33
(iii) Observations	34
(iv) Techniques	43
C. Ticks	48
Experiments on rodents	50
A. <u>Babesia rodhaini</u> in mice	50
Introduction	50
Experiments	51
a) Characteristics of infection	51
b) Titration of inoculum dose	52
Discussion	57
B. <u>Babesia rodhaini</u> in rats	60
Introduction	60
Experiments/	60

## Experiments

a) The adaptation of <u>B. rodhaini</u> to rats and the characteristics of infection therein	61
Discussion	63
b) Duration of infection in rats	64
(i) Determined by splenectomy	64
(ii) Determined by subinoculation	65
(iii) Comparison of splenectomy and subinoculation as means of detecting latent infection	67
(iv) Titration of infected rat blood into mice	70
(v) A further series of subinoculations	73
(vi) Effect of passage	74
(vii) Effect of host age	76
(viii) A final experiment	77
Discussion	79
c) Immunity in recovered rats	82
Discussion	83

C. Babesia microti in mice 86

## Introduction 86

## Experiments 87

a) Characteristics of infection	87
b) Persistence of infection and immunity	88
c) Effect of B-methasone in recovered mice	90

	Page
Discussion	92
Experiments on cattle	97
Introduction	97
A. The evaluation of some important techniques	99
a) Acridine orange staining	99
b) Plasma cortisol determination	104
c) Indirect Fluorescent Antibody (I.F.A.) test	106
B. <u>Babesia divergens</u>	117
Introduction	117
Observations on the primary infection of cattle	119
a) Splenectomised calves	119
b) Non-splenectomised cattle	128
c) The I.F.A. response	129
Discussion	131
Observations on chronic parasitaemias and some factors which might influence them	139
a) Pilot experiment	139
b) Experiment 1	143
c) Experiment 2	147
Discussion	151
C. <u>Babesia bigemina</u>	162
Introductory review of bovine babesiosis in Nigeria	162
The isolation of a Nigerian strain	166

	Page
Introduction	166
a) The attempted isolation from ticks	168
b) The attempted isolation from indigenous cattle	170
Discussion	172
The purification of a Nigerian strain	173
Discussion	177
Observations on the primary infection of cattle	178
a) Splenectomised calves	178
b) Non-splenectomised cattle inoculated with an impure isolate	182
c) Non-splenectomised cattle inoculated with a pure isolate	186
d) The I.F.A. response	191
Discussion	193
Observations on chronic parasitaemias and some factors which might influence them	200
a) Experiment 1	200
b) Experiment 2	204
c) Experiment 3	206
Discussion	209
The occurrence and effects of natural infection around Vom	213
Observations	213
Discussion	215

Concluding discussion	218
Acknowledgments	229
References	230
Appendices	262

Note: some terms which are used in the text are defined in Appendix 2. Figures presented thus:  $23.1 \pm 2.5$ , are arithmetic means and standard deviations respectively, unless otherwise stated. Personal communications cited in the text e.g. J. Smith, pers. comm., relate to 1975.

## SUMMARY

The thesis describes observations carried out on infections caused by various Babesia spp. in mice, rats and cattle with particular reference to a study of parasitaemias after acute infection.

Of mice infected with Babesia rodhaini, 95% died. The mortality rate was not affected by decreasing the number of parasites inoculated, although the time to death was proportionately increased.

Rats infected with B. rodhaini usually survived after varying degrees of illness and moderate parasitaemias. Parasitaemias fell rapidly from peak levels to latency. In most rats persistent infections were not detectable by subinoculation or splenectomy beyond ten days after the onset of latency. Subinoculation was shown to be an extremely sensitive means of detecting infection. Rats which naturally effected a sterile cure were immune to homologous challenge for at least a year.

All mice infected with B. microti recovered. The primary parasitaemia declined in a series of progressively smaller recrudescences. Persistent parasitaemias remained patent in some mice for as long as a year. The magnitude and occurrence of chronic patent parasitaemias did not decrease with increasing time from infection. B-methasone administration had no significant effect on chronic parasitaemias.

Babesia divergens infection in splenectomised calves was characterised by moderate parasitaemias accompanied by fever, anaemia, haemoglobinuria and some deaths. The decline in packed cell volume (P.C.V.) was associated with the fall in parasitaemia rather than with its rise. There was a positive correlation between the degree of anaemia and the level of maximum parasitaemia. In non-splenectomised cattle, infection produced only low and transient parasitaemias.

Chronic parasitaemias were monitored daily in splenectomised calves by the examination of thin blood smears stained with acridine orange. In this way,  $10^5$  erythrocytes in each smear could be quickly examined. Parasitaemias fluctuated with frequent and regular spontaneous recrudescences. The pattern of recrudescences was not affected by the administration of B-methasone or adrenocorticotrophic hormone (A.C.T.H.), but recrudescences were provoked by beginning to handle and sample the cattle. Relapse parasitaemias were of low magnitude and were not accompanied by anaemia or other clinical signs.

The indirect fluorescent antibody (I.F.A.) test was used to determine the antibody titres in primary and chronic infections. The response of splenectomised calves and non-splenectomised cattle was similar. After infection, antibodies were not detectable until maximum parasitaemias were reached. Titres continued to rise as parasitaemias fell, maximising 30-40 days after infection.

There was no difference between mean maximum titres of splenectomised and non-splenectomised cattle. Titres persisted for at least 500 days. Changes in titre were detected neither in association with recrudescent parasitaemias nor with A.C.T.H. and B-methasone administration.

In Nigeria, B. bigemina was isolated from latently infected indigenous cattle. The original isolate contained six other species of haemoparasite. These contaminants were eliminated by a series of rapid passages in splenectomised calves, and a pure isolate of B. bigemina was obtained. Six non-splenectomised adult steers were infected with the impure isolate of B. bigemina, and ten similar cattle were infected with the pure isolate.

In splenectomised calves, moderate parasitaemias caused severe disease with fever, anaemia, haemoglobinuria and death in 3/5 animals. The onset of anaemia was associated with the crisis of parasitaemia rather than with its rise. In non-splenectomised steers, infection caused low parasitaemias and minimal symptoms of disease with a variable degree of anaemia. The depression of P.C.V. was correlated positively with the magnitude of maximum parasitaemia.

The I.F.A. response was similar in splenectomised and non-splenectomised cattle. Antibody was first detected between one and five days after maximum parasitaemia. Maximum titres were not significantly different in splenectomised and non-splenectomised cattle and were



reached between 16 and 40 days after infection.

In the six steers infected with an impure isolate of B. bigemina, chronic parasitaemias were monitored for 115 days. Anaplasma marginale was frequently patent during this time and a reciprocal relationship was noted between it and B. bigemina, with the former parasite apparently dominant. Falls in A. marginale parasitaemia were followed by recrudescences of B. bigemina. B-methasone administration was followed by B. bigemina recrudescence parasitaemias in 2/6 animals. In the other four animals a response appeared to be abolished by the presence of A. marginale.

In the ten steers infected with a pure isolate of B. bigemina, parasitaemias generally remained latent after primary parasitaemia. Relapse parasitaemias were provoked in 8/8 animals treated with B-methasone although a pre-existing infection of A. marginale in some cattle inhibited this response. Two animals showed minor recrudescences of B. bigemina after A.C.T.H. treatment.

The chronic B. bigemina parasitaemia in one splenectomised calf was observed for 79 days. Throughout this period the parasitaemia remained patent and fluctuated periodically. The feeding of non-infected ticks did not affect the parasitaemia, but B-methasone treatment was followed by a major recrudescence which caused a moderate fall in P.C.V.

Exotic cattle imported to Nigeria were regularly bled

for serum over a 14 month period during which they were exposed to limited natural tick infestation. Five out of fifty cattle became infected with B. bigemina as was evidenced by a change in their I.F.A. status, but none was clinically ill nor was the milk yield affected.

A similar series of studies is being carried out in the laboratory of the author at the University of Liverpool. The results of these studies will be published in the near future. The author is grateful to the University of Liverpool for the facilities provided for this work. The author is also grateful to the University of Liverpool for the facilities provided for this work. The author is also grateful to the University of Liverpool for the facilities provided for this work.

A similar series of studies is being carried out in the laboratory of the author at the University of Liverpool. The results of these studies will be published in the near future. The author is grateful to the University of Liverpool for the facilities provided for this work. The author is also grateful to the University of Liverpool for the facilities provided for this work. The author is also grateful to the University of Liverpool for the facilities provided for this work.

Because of the wide-ranging nature of the studies described, an all-embracing literature review will not be presented. A few general points will be touched on briefly and some areas of specific relevance will be discussed more fully. Much of the literature will be mentioned later, where appropriate.

The Ixodidae spp. are protozoan parasites of vertebrates and ticks. Their classification has been, and still is, the subject of much debate (Rick, 1963; Levine, 1973). At the present time, Friedhoff, Avari & Brooksbury

## INTRODUCTION AND LITERATURE REVIEW

The thesis describes a series of linked studies of various Babesia spp.-host systems, beginning with an examination of rodent infections with particular reference to the nature of recovery from primary infection and to their suitability and use as models for later work. This is followed by an account of studies of Babesia divergens infections of cattle and in particular of the chronic parasitaemias in recovered animals. These investigations were conducted at the Centre for Tropical Veterinary Medicine, Easter Bush near Edinburgh.

A similar series of studies is then described, involving B. bigemina in cattle. This was carried out at the National Institute for Veterinary Research, Vom, Nigeria, whilst the author was a member of the C.T.V.M./Vom Collaboration Project.

Because of the wide-ranging nature of the studies described, an all-embracing literature review will not be presented. A few general points will be touched on briefly and some areas of specific relevance will be discussed more fully. Much of the literature will be introduced later, where appropriate.

The Babesia spp. are protozoan parasites of vertebrates and ticks. Their classification has been, and still is, the subject of much debate (Riek, 1968; Levine, 1973). At the present time, Friedhoff, Zwart & Brocklesby

(1974) state that there is general agreement that the family Babesiidae (Poche, 1913) lies within the class Piroplasma (Levine, 1961) of the subphylum Apicomplexa (Levine, 1973), but that the classification below family level requires clarification. In the present thesis all the species mentioned are regarded as members of the genus Babesia (Starcovici, 1893), although some of them (e.g. B. microti and B. rodhaini) have been quite commonly ascribed to another genus, Nuttallia (França, 1910). Taxonomic arguments apart, it has only just been pointed out (Peirce, 1975) that Nuttallia (França, 1910) was pre-occupied by Nuttallia (Dall, 1898) for molluscs, so that this name will have to be abandoned for piroplasms.

The small Babesia spp. parasite of cattle which was encountered in Nigeria is referred to as B. bovis (Babes, 1888). Some of the literature cited in this thesis refers to B. argentina (Lignières, 1903), by which name the small Babesia spp. parasitising cattle in Australia has always been known. There is no doubt that B. argentina and B. bovis are very similar. Riek (1968) regarded them as being synonymous and current opinion favours that view (P. Leeflang, pers. comm.). However the name B. argentina will be retained when quoting original papers in which it was used.

In the mammalian host Babesia spp. parasitise cells of the erythrocytic series. In erythrocytes, organisms multiply by budding or binary fission and invade non-

infected cells. This phase may be accompanied by clinical illness and haemolytic anaemia. The breakdown of erythrocytes releases haemoglobin which is excreted in urine and gives the disease its colloquial name of 'redwater'.

The degree of anaemia may be disproportionate to the parasitaemia (Neitz, 1938). This fact has stimulated some workers to seek evidence for the role of auto-immune phenomena in the pathogenesis of the disease (Schroeder, Cox & Ristic, 1966). It is established that pharmacologically active substances are released by the breakdown of erythrocytes (Goodwin & Richards, 1960), and disseminated intravascular coagulation has been incriminated as yet another component of the disease (Dolan, 1974).

Babesia spp. parasitise a wide variety of mammals and cause a variable but significant amount of disease in cattle, horses, sheep, dogs and, to a lesser extent, pigs, on a global scale. Consequently they have been the subject of much veterinary research. This research has been given a new impetus by the emerging reputation of babesiosis as a zoonosis (Anon, 1976).

The parasites are transmitted to their vertebrate hosts by ixodid ticks. In 1893, Smith & Kilborne reported that Babesia bigemina was transmitted by Boophilus annulatus. This was a momentous discovery. It was the first time that an arthropod vector was shown to transmit an infectious agent and it stimulated the search for vectors of many other diseases. Several species of

tick are now known to be capable of transmitting Babesia spp. (Neitz, 1956). Ticks serve as true biological vectors. The parasite undergoes a developmental cycle in the tick and may be transmitted either from stage to stage or through the egg. The life-cycle in the tick has been worked out for several Babesia spp. in their respective vectors and is reviewed by Riek (1968), and more recently and briefly by Mahoney (1972).

Infections with Babesia spp. parasites classically persist for long periods in the vertebrate host. During this time the host is generally immune to re-infection with the same species. Sergeant, Parrot & Donatien (1924) called this state of immunity and co-existent infection 'premunity'. Until relatively recently it was believed that persistence of infection was necessary for the maintenance of immunity but studies involving the sterile cure of infections by chemical means and observations on naturally recovered animals have shown quite clearly that the state of premunity is succeeded by a period of sterile immunity (various authors, cited in Callow, McGregor, Parker & Dalgliesh, 1974).

During the state of premunity parasites can only infrequently be detected in blood by the examination of conventional blood smears. This fact has caused speculation about what happens to parasites in chronically infected animals. In spite of this interest, there is no conclusive evidence that exo-erythrocytic stages of the



parasite exist in the mammalian host. Riek (1968) cites reports by Ivanic (1942) and Kolabsky (1954) that multiplication takes place in internal organs, but these have not been confirmed.

The continued existence of parasites in the blood, at sub-patent levels, is proved by the ability of blood from premune animals to infect susceptible animals. This technique of subinoculation may, however, be negative even when animals are later proved to be infected (e.g. in Joyner & Davies, 1967). In such circumstances the failure of subinoculation to prove the presence of infection, like the failure of visual detection methods, is most likely to be a quantitative artifact. It is possible to subinoculate only a fraction of the blood volume of a donor animal, and, together with the fact that the minimum infective dose for a recipient might be considerably in excess of one parasite, it is feasible that low numbers of parasites might exist, undetected, in the circulating blood. This argument applies to a much greater extent when visual detection methods are employed. If  $10^5$  erythrocytes (the maximum number which it is practicable to examine in a thin blood smear) are examined, then parasite densities of less than  $1/10^5$  will not be observed. Calculation shows that for an adult cow this means that as many as some  $10^8$  parasites could freely exist undetected in the circulation. These considerations counter the argument that, because parasites cannot be

detected in the blood, they must be somewhere else. Nevertheless the possibility of exo-erythrocytic forms of the parasite existing cannot be totally dismissed in view of what is known of the natural history of many other Protozoa such as Plasmodium and Theileria spp., which have tissue as well as blood stream forms, and of the fairly closely related parasite Nuttallia dani which resembles the Theileriidae in undergoing schizogony in internal organs as well as having an intro-erythrocytic phase (Tsur, Hadani & Pipano, 1960).

Although Babesia spp. in all probability only parasitise erythrocytes in the vertebrate host, the density of parasitaemia may not be uniform throughout the circulatory system. Certain species have a marked tendency to concentrate in capillary beds - most notably B. bovis in cerebral capillaries (Callow & Johnston, 1963) and B. berbera in blood vessels of the brain and kidney (Goldman, Pipano & Rosenberg, 1972).

The duration of chronic infection is long and is generally measured in terms of years rather than months. B. bigemina has been recorded in a bovine host twelve years after infection (Neitz, 1956) but the more usual term is probably less than two years (Mahoney, Wright & Mirre, 1973). This period may vary from species to species - e.g. B. argentina is likely to persist for at least four years after a single infection (Mahoney, Wright & Mirre, 1973) and B. divergens probably persists for 2-3



years but may be present in splenectomised cattle as much as 6 years after a single infection (Joyner & Davies, 1967).

Because of the need to detect carrier animals which have infections at very low levels, a variety of means of detecting infection have been devised and applied. Some of these have been reviewed by Sargent (1963), Ewing (1966) and Jungmann (1966). They can be categorised into 'direct' and 'indirect' methods, in which the former detect parasites and the latter detect antibodies, the presence of which may or may not be related to the presence of parasites.

The simplest direct method is to examine blood smears. Conventional examination of Giemsa-stained thin blood smears has a very limited sensitivity. With care and patience no more than  $10^5$  erythrocytes can be examined. Alternative staining methods may improve the efficiency of thin smear examination. Bishop & Adams (1973) found that the addition of alkyl phenoxy polyethoxy ethanol (A.P.P.E.) to Giemsa solution improved the quality of staining. A completely different stain, acridine orange, has been used by Winter (1967) to stain thin blood smears. He reported that the use of this stain enabled parasites to be detected in blood at far lower concentrations than was possible using Giemsa-stained thin and even thick smears.

Mahoney & Saal (1961) described the use of thick

blood smears for the detection of Babesia spp. parasitaemias. Many more erythrocytes can be scanned in a thick smear than in a thin one, but the latter has the advantage that parasitaemia ratios can easily be derived since erythrocytes can be counted. Using thick smears, parasitaemias can only be quantified either by relating parasite counts to leucocytes and performing leucocyte counts (two procedures, which inevitably increases error), or by counting parasitaemias in the whole of a smear prepared from a known volume of blood. This latter can be very time consuming but has the advantage that an absolute parasitaemia is obtained. The use of thick smears has been extensively and successfully applied particularly by Australian workers whose patience must be admired. For the detection, as opposed to the quantification, of parasites thick smear examination is undoubtedly the best of direct visual methods.

A number of other counting techniques which give absolute parasitaemias have been described. These can be especially useful when assessing parasitaemias in recovering animals when the erythrocyte count is altering day by day. Acridine orange was applied by Winter (1968) in a direct counting technique using a counting chamber. More recently, Parker (1973), has devised another direct counting technique without a counting chamber. Both these methods are only applicable to relatively high parasitaemias and are not claimed to be of assistance in

detecting low parasitaemias.

In the case of parasites which concentrate in particular capillary beds, knowledge of these sites of concentration can be exploited if accessibility allows. These conditions apply to B. argentina infections and Johnston & Callow (1963) have described a brain biopsy technique which enables brain smears to be prepared from live animals. This greatly enhances the chances of observing B. argentina in a latently infected host (Callow & Johnston, 1963).

B. argentina tends to exist in higher densities in capillary than in venous blood, so that blood smears made from scraping the tail tip may contain up to ten times more parasites than blood smears made from venous blood at the same time (Hoyte, 1971).

Another technique of experimental interest is the possibility of temporarily elevating levels of parasitaemia by the administration of immunosuppressants. Callow & Parker (1969) found that this revealed B. argentina at patent levels in latently infected animals.

The possibility of 'elevating' parasitaemias in vitro by concentration methods has not received much attention. Erythrocytes infected with B. canis and with B. caballi will concentrate at the top of packed red cells when infected blood is centrifuged (Watkins, 1962; Hirsch, Hickman, Burkholder & Soave, 1969). This phenomenon has not been reported for B. bigemina, although work recently

in Nigeria (S.A. Ajayi and J.R. Best, pers. comm.) suggests that the parasite will also concentrate in this manner. This could be a very simple and effective aid to the detection of latent infection.

B. argentina can be concentrated by exploiting the fact that infected cells resist lysis in hypotonic solutions better than non-infected cells. Mahoney (1967) has developed this as a means of concentrating infected cells for antigen production, but whether it could be useful as an aid in detecting low parasitaemias is doubtful. There are conceivable limitations such as the fact that it is time consuming and may not be applicable to some species e.g. B. bigemina (Mahoney, 1967).

Subinoculation is a valuable detection method which involves the transfusion of blood from an animal suspected of harbouring parasites to one known to be susceptible to infection and in which patent parasitaemias will be produced if parasites are present in the subinoculum. The potential sensitivity of this method is very high and is directly related to the volume of blood subinoculated and to the number of parasites needed to patently infect the recipient. Using splenectomised recipients, the minimum infective dose may be very small. Whilst still not definitive if negative results are obtained, of all the detection methods subinoculation enables the absence of infection to be determined with the greatest confidence.

Latently infected animals will frequently suffer relapse parasitaemias after splenectomy (Miessner, 1931; Sergeant, Donatien, Parrot & Lestoquard, 1945; Phillips, 1969b and others). This technique is obviously not suitable for diagnostic purposes but can be useful experimentally.

It can be seen that all the methods discussed so far vary in their sensitivity, practicability and economic feasibility. The particular situation in which an investigator finds himself will determine the choice of method after consideration of the merits of each. The investigations of chronic parasitaemias in cattle described in this thesis required a reasonably simple and efficient detection method which could be applied daily for prolonged periods to groups of cattle, which would allow quantification of parasitaemias and which would be adequately sensitive. The examination of acridine orange stained thin smears was selected for this purpose.

The 'direct' detection methods can prove definitively that an animal is still infected with parasites. 'Indirect' detection methods cannot do this since they detect antibodies and not parasites. Nevertheless these serological tests are included in this discussion since they provide the best detection methods applicable on a large scale to epidemiological research and to babesiosis control and eradication programmes.

The relationship between the presence of antibodies

and the presence of parasites varies from test to test. Mahoney (1962) developed a complement fixation (C.F.) test and found that titres to B. bigemina disappeared between 4-7 months after infection. Since this time is much shorter than the expected duration of infection by this parasite, a proportion of animals negative to the test may in fact be infected. Mahoney (1964) found this to be the case; although the test did give positive results in some cattle which were negative on thick smear examination, many animals which were serologically negative were proved, by subinoculation, to be infected.

Antibodies detected by the indirect fluorescent antibody (I.F.A.) test persist for longer periods. Thus Ross & Löhrr (1968) detected antibody 18-24 months after cattle had been infected with B. bigemina, and Johnston & Tammemagi (1969) found that I.F.A. titres in B. argentina infected cattle persisted for 13 months in association with latent infection, whereas C.F. titres had in some cases disappeared. Whilst I.F.A. titres are not necessarily associated with protective immunity, they are reasonably well associated with the presence of infection. After the sterile cure of B. argentina infections by drugs, I.F.A. titres disappeared within six months (Callow, McGregor, Parker & Dalgliesh, 1974) although cattle were still immune to challenge at this time.

A capillary agglutination test for B. bigemina was described by Löhrr & Ross (1969). Titres persisted for



long periods and appeared to relate well to the persistence of infection. The test was easy to perform but the quality of the antigen was critical to the working of the test and could be variable.

Several other serological tests have been described for Babesia spp., including direct (Todorovic, Ferris & Ristic, 1967) and indirect (Curnow & Curnow, 1967) haemagglutination, gel-precipitation (Ristic & Sibinovic, 1964) and slide-agglutination (Curnow, 1973a) tests. These tests all have particular merits, but some require fairly sophisticated methods of antigen preparation which might limit their use in the tropics. Others are strain specific which is disadvantageous for screening procedures.

After a consideration of these tests, the I.F.A. test was selected for application in the present studies. The preparation of antigen is straightforward, the test relates reasonably well to the presence of infection, and it will distinguish adequately between Babesia species but not between strains (Ludford, 1969; Leeftlang & Perié, 1972; Johnston, Pearson & Leatch, 1973). By titration it was intended to use the test to assess antibody levels during acute and chronic infection.

During the period of chronic parasitaemia following primary infection (these states have been respectively called babesiasis and babesiosis, Mahoney, 1972), the parasite and the host co-exist in a state of dynamic

equilibrium. This relationship for the most part is unobserved, since parasitaemias are likely to be sub-patent, but, immediately following primary infection, recrudescent parasitaemias of lesser magnitude than primary parasitaemias may be seen (Davies, Joyner & Kendall, 1958; Barnett, 1965; Allen, Frerichs & Holbrook, 1975). Over a longer period patent recrudescent parasitaemias may occur spontaneously (Smith & Kilborne, 1893; Dodd, 1910; Legg, 1933) and sometimes frequently enough to exhibit a degree of periodicity (Ewing, 1965; Mahoney, 1972). Recrudescent parasitaemias of this type in B. argentina infected cattle have recently been shown to involve antigenic variants of the initial infecting strain (Curnow, 1973b). The relapse parasitaemias were not associated with clinical disease.

Clinical relapses in chronically infected animals are a feature of Babesia spp. infections much referred to in monographs on babesiosis. A search of some standard texts has produced the extensive list in Fig. 1. In spite of these citations and the fact that relapses were associated with intercurrent infection as long ago as 1899 (Nicolle & Adil Bey, cited by Wenyon, 1926), very few of these relationships have been experimentally investigated. The examples in Fig. 1 are presumably drawn from field observations, in which the possibility of re-infection can, in many cases, not be ruled out. Some of these observations may therefore pertain to situations of



Fig. 1

A list, extracted from a selection of texts, of conditions  
said to provoke relapses of clinical babesiosis

Parturition	Richardson & Kendall (1963)
	Henning (1956)
Inclement weather	Richardson & Kendall (1963)
	Henning (1956)
Physical stress	Henning (1956)
Starvation	Blood & Henderson (1968)
Rinderpest immunisation	Richardson & Kendall (1963)
Intercurrent infection	
e.g. foot and mouth disease	Levine (1961)
distemper	" "
East Coast Fever	Henning (1956)
mastitis	" "
paratyphoid	" "
verminosis	" "
anaplasmosis	Blood & Henderson (1968)
Severe tick infestation	Henning (1956)

challenge during stress, rather than to recrudescences of latent infection. Moreover, some of the examples cited refer to animals which have been moved considerable distances (for instance to vaccination points) and in the process have been exposed to ticks in a foreign locality. There is a considerable body of evidence that strains exist within species of Babesia which will not completely cross-protect and this has been demonstrated in the laboratory as well as in the field (Riek, 1968; Rogers, 1971b). The effects of challenge by a heterologous strain could be exacerbated by stress to an extent where frank disease was caused.

In view of these considerations, it is not clear how frequently genuine recrudescent parasitaemias cause disease in the field. With the notable exception of splenectomy which will cause clinical relapses (Miessner, 1931, and others), there have been very few studies of relapse provocation in babesiosis. Such studies as have been conducted have mainly investigated the effects of corticosteroids on chronic infections. In cattle infected with B. argentina and B. bigemina (Callow & Parker, 1969 and Hoffman, Schein & Müller, 1971, respectively) corticosteroid administration was succeeded by non-clinical relapse parasitaemias, although Young & Cox (1971) found that chronic B. microti parasitaemias were only occasionally affected. The results obtained by Callow & Parker (1969) prompted them to suggest that stress may

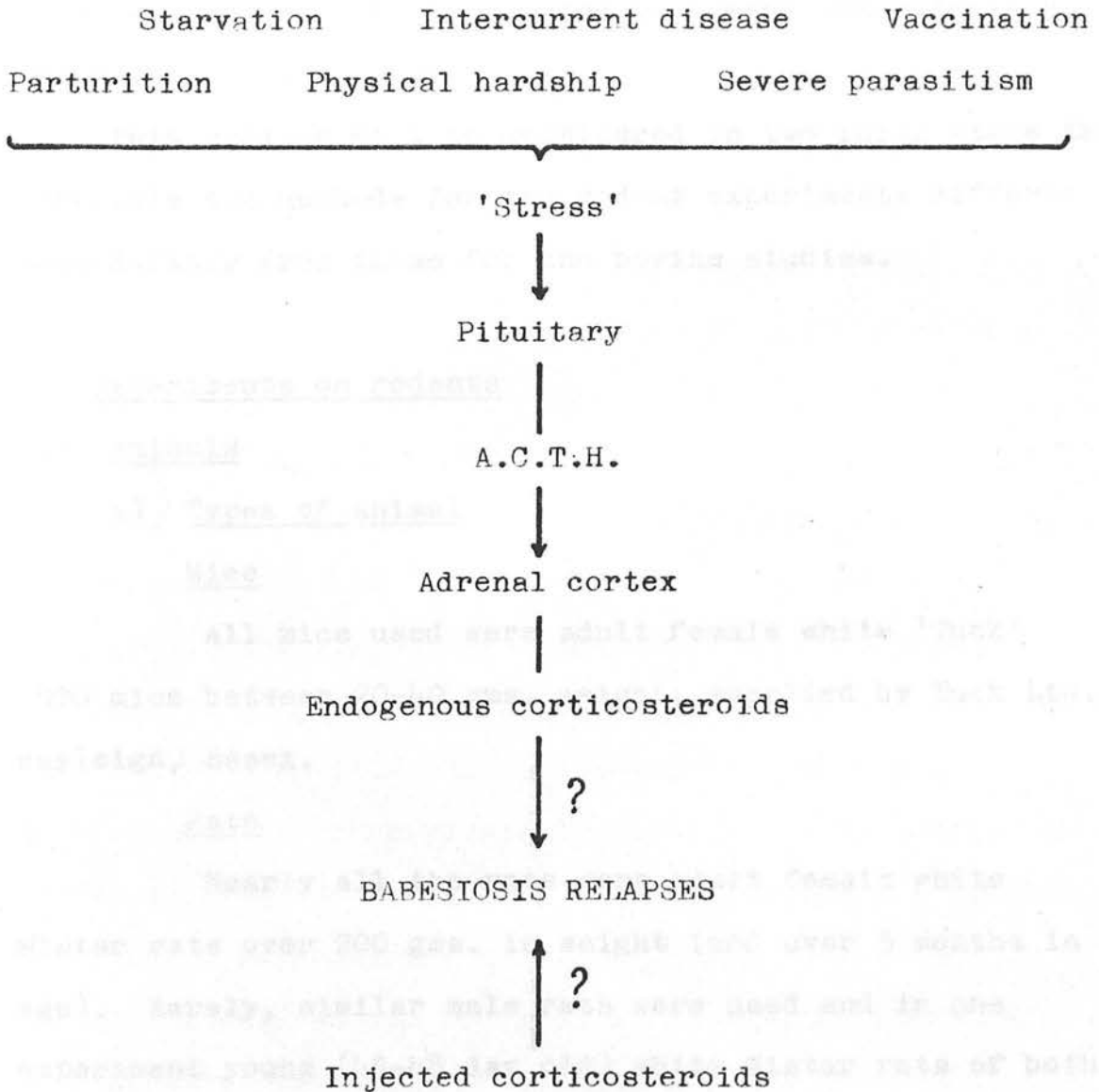
precipitate natural relapses because of the elevation of endogenous corticosteroids associated with it.

The very varied conditions which are said to provoke Babesia spp. relapses and which are listed in Fig. 1 are linked by the fact that all are 'stressors' (as defined by Fraser, Ritchie & Fraser, 1975). In his classic studies of stress, Selye (1936 and 1956) recognised that the variety of responses of the body to different stressful situations was limited. Just as Cannon (1929) had rationalised the 'fight and flight' responses involving the adrenal medulla, Selye unified the body's response to more prolonged stress as a function of the adrenal cortex which he called the General Adaptation Syndrome (G.A.S.).

Whilst Selye's approach may be regarded by contemporary authors (Fraser, Ritchie & Fraser, 1975) as too simplistic, it serves to link the many varied situations in which Babesia spp. relapses are said to occur. Taken in conjunction with the known immunosuppressive activity of corticosteroids it provides the hypothesis represented in Fig. 2. This hypothesis proposes that; because injected corticosteroids provoke parasitaemic recrudescences, then babesiosis relapses following 'stress' are probably a direct result of raised endogenous corticosteroid levels. It provides the framework around which the experiments on chronic Babesia spp. parasitaemias were planned.

Fig. 2

A hypothetical causal relationship between stress and babesiosis relapses



? - denotes relationships which are investigated in the present thesis

## MATERIALS AND METHODS

Because experiments were performed on a variety of animals, with different parasites and in Africa as well as in Edinburgh, it has not been possible to standardise all materials and methods. Instead it has been necessary, especially in Africa, to use the equipment and materials at hand.

This section will be considered in two parts since the materials and methods for the rodent experiments differed considerably from those for the bovine studies.

### A. Experiments on rodents

#### (i) Animals

##### a) Types of animal

##### Mice

All mice used were adult female white 'Tuck' CDTO mice between 20-40 gms. weight, supplied by Tuck Ltd., Rayleigh, Essex.

##### Rats

Nearly all the rats were adult female white Wistar rats over 200 gms. in weight (and over 3 months in age). Rarely, similar male rats were used and in one experiment young (45-48 day old) white Wistar rats of both sexes were used. All the rats were supplied by the Edinburgh University Centre for Laboratory Animals (E.U.C.L.A.).

b) Feeding and management of rodents

Mice were kept in wire-topped plastic cages 32 cms. long x 13 cms. wide x 12 cms. deep. Not more than six mice were kept in one cage. Woodshavings were provided as bedding. Water was available ad libitum and proprietary rodent nuts were provided.

Cages were kept in racks in centrally heated, well ventilated rooms at 70° F. Mice and rats were kept in separate rooms.

The management and feeding of rats were identical except that they were kept in larger cages, 54 cms. x 34 cms. x 19 cms., each containing not more than five rats.

(ii) Babesia spp.

a) Babesia rodhaini

This was a mouse strain which had been kept as a stabiliate (designated TREU 812) at -79°C at the C.T.V.M., Edinburgh. The strain had originally been provided by Mrs. K.M.G. Adam, Zoology Department, University of Edinburgh. The stabiliate was inoculated into mice and maintained thereafter by weekly passage for the duration of the experimental work. In this way, parasitaemic blood was readily available at all times.

For the infection of rats, a rat-adapted strain of this parasite was derived by infecting rats with B. rodhaini infected mouse blood. Over several passages in rats, a strain was selected which consistently produced uniform infections. This rat-adapted strain of B. rodhaini

was maintained thereafter by weekly passage in rats and also preserved as stablate on different occasions.

b) Babesia microti

The King's College strain, originally isolated from the short-tailed vole, Microtus agrestis, and adapted to mice by Cox & Young (1969), was preserved as stablate (TREU 1122) at the C.T.V.M. after about 120 passages in mice. This stablate was used for the initial infections of mice in this work and the strain was thereafter maintained by weekly passage.

(iii) Observations

a) Parasitaemia

Blood samples were invariably taken in the morning and were obtained by snipping the tail tip with sterile scissors. Levels of parasitaemia were expressed as the percentage of infected to total erythrocytes (i.e. no account was taken of the infection of individual erythrocytes by more than one parasite) discerned in thin blood smears fixed in methanol and stained with Giemsa (see section B for staining method).

The actual number of red blood cells examined varied according to the general level of parasitaemia - as follows:

greater than 10%	-	200 r.b.c.s examined
between 1% and 10%	-	500 r.b.c.s examined
between 0.1% and 1%	-	1,000 r.b.c.s examined
less than 0.1%	-	10,000 r.b.c.s examined

High parasitaemias were counted using a x100 oil immersion objective, whilst with very low parasitaemias a x54 oil immersion objective was used in order to facilitate the examination of a large number of erythrocytes.

b) Haemoglobinuria

The examination of urine for haemoglobinuria was sometimes used to provide rapid presumptive evidence of Babesia rodhaini infection - most notably in mice subinoculated with blood from rats suspected of harbouring latent parasites. In some cases blood smears were also examined, but, as a general rule, mice which developed haemoglobinuria following inoculation with blood either known to be, or suspected of being, infected with B. rodhaini, were regarded as having suffered from babesiosis. Animals which died after a period of haemoglobinuria were regarded as having died from babesiosis. Any mice which died without haemoglobinuria were not regarded as having died from the effects of babesiosis.

No other haemoparasites which might have caused haemoglobinuria were observed at any time in the hundreds of blood smears examined from the rodents used throughout this work.

In order to ascertain if haemoglobinuria was present, mice were individually examined. Mice were grasped at the nape of the neck between thumb and forefinger and held extended on their backs in one hand with the little finger gripping the tail. In this position the bladder could be



palpated through the abdomen by the thumb and forefinger of the other hand and by gentle squeezing, urine could be expressed through the urethra.

(iv) Techniques

a) Passaging

Donor mice were anaesthetised with ether, the axillary blood vessels were exposed by dissection and the subclavian vein was severed. Blood was transferred by Pasteur pipette into a test-tube containing an anticoagulant solution. This consisted of phosphate buffered salt solution pH 8.0 (ABP 8.0 - see Appendix 1) with heparin added to 50 i.u./ml.

Nine volumes of blood were added to one volume of anticoagulant solution. For routine passaging, this mixture was diluted tenfold using solution ABP 8.0. The diluted blood was immediately inoculated into recipient animals. A 25g. x  $\frac{5}{8}$ " needle was used for this purpose.

Donor rats were similarly anaesthetised, but blood was withdrawn from the heart, as described below.

The volume of diluted blood inoculated was 0.1 ml., which of a 1 in 10 dilution of blood from a heavily parasitised mouse or rat, amounted to  $10^7$  or  $10^8$  infected erythrocytes. This inoculum dose conveniently allowed a passage interval of about one week.

The intraperitoneal route of inoculation was used throughout.

b) Subinoculation

This technique was used as a means of detecting latent infections in rats. Rats were anaesthetised, using ether inside a closed jar, then removed when anaesthesia was complete and placed on their backs. Blood was withdrawn from the heart, using a 25g. x  $\frac{5}{8}$ " needle and a 1 ml. syringe which had previously had heparin solution sucked into, then blown out of it. Sufficient heparin remained in the syringe to prevent coagulation of the 0.5 ml. of blood which was withdrawn.

The blood was immediately inoculated into two mice, each of which received 0.25 ml. intraperitoneally.

Rats could be safely bled in this way, sometimes on several occasions. The rare deaths which occurred were attributable to the effects of ether anaesthesia rather than to haemorrhage.

c) Titration

This was a combination of the previous two techniques, in which very precise dilutions of donor blood were used to inoculate groups of mice.

Blood was collected from the donor animals using just sufficient anticoagulant (di-sodium edetate, E.D.T.A.) to prevent coagulation. A sample of this blood was taken and thin smears were prepared from which parasitaemias could be calculated and a red blood cell count was made either by using an electronic particle counter ('Coulter Counter' Model FN, Coulter Electronics Ltd., Dunstable, Beds.), or

by use of a Neubauer haemocytometer. From the values of parasitaemia and red cell count, the density of parasitised cells per unit volume of donor blood was calculated.

The bulk of the donor blood was used to make a series of tenfold dilutions in solution ABP 8.0 in test tubes held in an ice bath. From these dilutions 0.1 ml. aliquots were inoculated intraperitoneally into mice.

The number of parasitised cells inoculated into each mouse at each dilution could be determined by simple calculation.

d) Cryopreservation

Although both B. rodhaini and B. microti were maintained by passage in rats and/or mice, on occasions infected blood was frozen to provide stabilates (Lumsden & Hardy, 1965). As cryoprotectant, glycerol was added to aliquots of parasitised heparinised blood to a final concentration of 7.5%. The blood was then heat-sealed into glass vials and stored at  $-79^{\circ}\text{C}$  in insulated cabinets containing solid carbon dioxide.

e) Splenectomy of rats

Rats were anaesthetised by ether or more usually by a single intraperitoneal injection of pentobarbitone sodium ('Nembutal', Abbott Laboratories, Sittingbourne, Kent) at 6 mgms./225 gms. weight about 30 minutes prior to the operation.

Anaesthetised rats were stretched on their right sides on a table and an area of about 2 sq. cms. between

the last rib and the iliac crest of the pelvis, just ventral to the transverse processes of the lumbar vertebrae, was shaved and cleaned with spirit. Using full aseptic techniques, a vertical incision, about 1.5 cms. long, was made just posterior to the last rib. The spleen was located with forceps and gently exteriorised. The splenic vessels were clamped and ligated with 2/0 chromic catgut, and the spleen was removed. The abdominal muscle wound was closed by continuous suture and the skin wound was closed separately with a single mattress suture, both with 2/0 chromic catgut. A streptomycin/penicillin cream ('Streptopen' - intramammary preparation, Glaxo, Greenford, Middlesex) was squeezed into the incision. This prevented wound abscesses which had occurred without its use in some earlier operations.

By this means, 33 rats were successfully splenectomised. Very few deaths occurred and these were usually associated with ether anaesthesia. Barbiturate anaesthesia was found to be very safe and convenient, and allowed the operator to perform unaided, and without the need to attend to anaesthesia.

## B. Experiments on cattle

### (i) Animals

#### a) Types of cattle

In Britain, Bos taurus steers of the Ayrshire and Jersey breeds were used. These had been purchased as

calves from babesiosis-free areas near Edinburgh and reared at the C.T.V.M. Most of these cattle were splenectomised before they were used in experiments.

In Nigeria, with the exception of one pure Fulani (Bos indicus) calf, all the experimental animals were Bos taurus/Bos indicus cross steers. More specifically they were either Friesian cross Fulani or Brown Swiss cross Wadara animals, in which the ratio of Bos taurus to Bos indicus in the genotypes was one or in most animals greater than one. This was reflected in the phenotypes; the cattle were not humped and bore the colourings of either the Friesian or Brown Swiss breed.

All these cattle were bred on the farm of the National Institute for Veterinary Research, Vom.

Some of these cattle were splenectomised prior to their use in experiments.

b) Feeding, management and housing of cattle

In Edinburgh, cattle were housed at all times. Ticks do not occur at Easter Bush Farm and so special precautions were not necessary to protect the cattle from them.

Bought-in cattle were sometimes infested with lice and this vector was implicated in the transmission of Eperythrozoon spp. infections amongst some of the cattle. For this reason cattle were, on occasions, dusted with a gamma benzene hexachloride powder (I.C.I. Louse and Insect Powder, I.C.I., Macclesfield, Cheshire) and treated with

an arsenical compound ('Spirotrypan Forte', Hoechst, Frankfurt, Germany) by intravenous injection.

Cattle were fed hay and rearing nuts ('Nutristart' Calf Cubes, Scottish Agricultural Industries, Edinburgh).

Ticks naturally infest the pastures in and around Vom, so that strict precautions were necessary to protect experimental animals from natural infestation.

Calves at Vom Farm were kept indoors from birth and after weaning, at about six weeks of age, were transferred to special animal quarters which had been rendered tick-proof.

Older cattle which had been maintained out of doors at Vom Farm, and which had been sprayed with acaricide once a week throughout their lives, were held before experiment in paddocks or open stables and sprayed once weekly. They were sprayed immediately prior to their transfer to the tick-proof quarters.

All experiments, except those involving the application of ticks, were conducted on animals kept in the tick-proof quarters. Calves were splenectomised inside the building. Cattle were kept in the tick-proof quarters for the duration of the experiments, but were not sprayed during this period.

Human access to the building was limited and controlled.

The tick-proof quarters were completely enclosed by solid walls and fine netting (Plate 1). The front

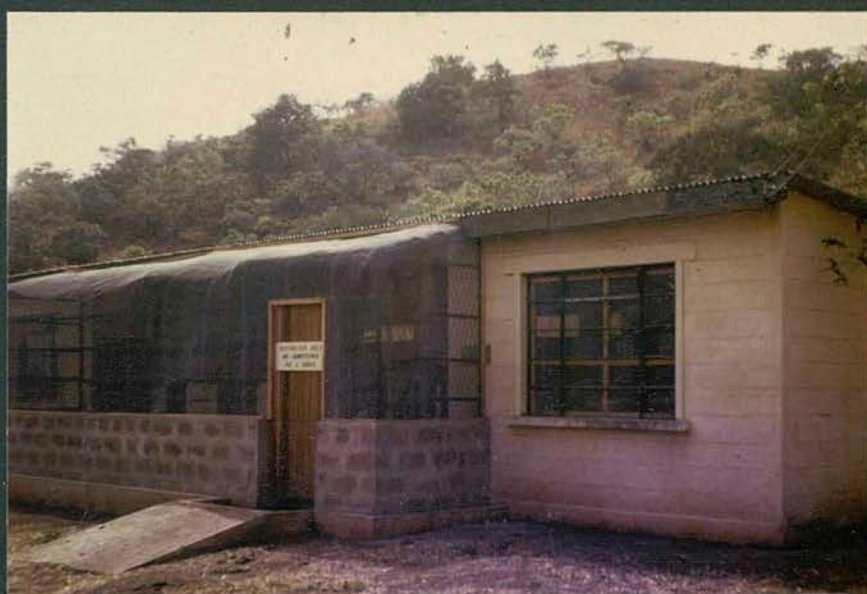
Plate 1

Tick-proof quarters

Plate 2

A pen in which ticks were applied  
to calves





of the animal house was surrounded by a small moat which ran into a foot bath under the door which was the only means of entry into the building. The bath and moat were kept filled with an acaricide solution ('Gamatox', Cooper, Berkhamstead, England) which was renewed every few days. Around the building, vegetation was cleared to leave a strip of bare earth some five yards wide, and beyond this was a stock-fence.

Cattle were fed the standard non-milking meal, made and supplied by the Animal Production Division of the N.I.V.R., Vom, together with cottonseed and hay.

The risk of introducing ticks into the tick-proof quarters in hay was recognised. At the Wellcome Veterinary Laboratories, Kabete, Kenya it has been the practice to fumigate hay with bromine before feeding it to animals involved in tick-borne diseases' experiments (W.G. McLeod, pers. comm.). The fumigation of hay was not adopted at Vom because of practical difficulties. Hay had invariably been stored for some time prior to its being fed. Barnett (1961) has observed Boophilus decoloratus ticks surviving in hay for several weeks, but he is of the opinion that hay stored for longer periods will probably be free of ticks. After the examination of hay bales, the Wellcome workers at Kabete also concluded that hay would be unlikely to be a source of ticks for housed stock. In practice, we had no cause to suspect the introduction of ticks to the tick-proof quarters in hay. Ticks were not found at any

time on cattle housed in those quarters nor was there any evidence throughout the study of the inadvertent transmission of haemoparasites between experimental cattle.

Experiments in which ticks were fed on cattle were conducted in separate quarters, adapted and used only for this purpose. Rectangular pens of about  $1\frac{1}{2}$  metres length by 1 metre width, were improvised in the back corners of a stone walled shed (Plate 2). The floor of the pens was hard laterite. Outside and parallel to the pen walls, vertical boards were set in the laterite to limit the creeping of dropped ticks. Calves were introduced to the pens directly from the tick-proof quarters. At the end of experiment, calves were sprayed with 'Gamatox' using a stirrup pump, and were then returned to the tick-proof quarters. The pens were then dismantled and thoroughly sprayed.

Human access to this building was limited, and different members of staff were used to tend those animals in the tick-feeding shed and those in the tick-proof quarters. The two buildings were set well apart.

(ii) Babesia spp.

a) Babesia divergens

This was used to infect cattle in Edinburgh. The Weybridge strain of B. divergens was kindly made available by Dr. L.P. Joyner. Fresh blood sent on ice from Weybridge was used to infect a splenectomised calf. Parasitised blood from this calf was stored as stabilate TREU



1081.

This stabilate was used by the author to infect calf no. 10. On the day this calf showed its maximum parasitaemia, a number of aliquots of blood were frozen down as stabilate TREU 1155. Most of the animals infected with B. divergens in the present study were infected with this stabilate.

b) Babesia bigemina

B. bigemina was isolated from latently infected Nigerian cattle. The isolation and purification of this strain are described in detail in later chapters.

Some cattle were infected with B. bigemina before other contaminating haemoparasites had been eliminated. Others were infected with a purified isolate of B. bigemina free from other haemoparasites and stored as stabilate C.T.V.M. 33.

(iii) Observations

a) Clinical

Animals on experiment were examined daily.

Rectal temperatures were measured with a standard clinical thermometer. Any obvious abnormalities, such as behavioural changes or anorexia were noted. A particular watch was made for haemoglobinuria during acute infection. Drops of urine could usually be found on the preputial hair if the animal had not been seen to urinate. The nature of the faeces was also noted.

Cattle which became ill were more thoroughly examined

by normal clinical methods. In some of these cases faecal samples were examined for the presence of coccidial oocysts and serum was retrospectively submitted for the detection of viral antibodies. Animals were weighed for the computation of drug dosages by means of a weighing tape, ('Weigh-band', Dalton Supplies Ltd., Henley-on-Thames, Oxon.).

b) Collection of blood

Blood was collected from either the external jugular or the coccygeal veins. Collections were made between 0900 and 1000 hours.

For most purposes 5 mls. evacuated tubes ('Vacutainers', Becton Dickinson, Ireland) containing 7 mgms. of disodium edetate (E.D.T.A.) as anticoagulant, together with a 20g. x 1" needle were used to withdraw and collect blood. If large volumes of blood were being collected for stabilate preservation or subinoculation, 50 mls. heparinised 'Vacutainers' were used. For serum collection, cattle were bled into plain 10 mls. 'Vacutainers'.

c) Parasitaemia

This was determined by the examination of thin blood smears stained with acridine orange and examined by fluorescence microscopy (see Observations for details of the latter).

It was calculated as the ratio of infected to total erythrocytes and no account was taken of the infection of individual erythrocytes by more than one parasite. To derive the parasitaemia ratio the actual number of red

blood cells examined varied depending on the general level of the parasitaemia as follows:

greater than 10%	-	200 r.b.c.s examined
between 1% and 10%	-	500 r.b.c.s examined
between 0.1% and 1%	-	10,000 r.b.c.s examined
less than 0.1%	-	100,000 r.b.c.s examined

High parasitaemias were counted using a x100 oil immersion objective and are expressed as percentage figures. The majority of parasitaemias described in this study were of the low-grade chronic type, at less than 0.1% level. These will sometimes be expressed as the number of parasitised cells per  $10^5$  erythrocytes. To derive this figure, either a x54 or a x40 oil immersion objective was used, and the appropriate number of fields was scanned to include  $10^5$  erythrocytes.

#### d) Haematology

In Edinburgh, an electronic particle counter was available. This measured red cell and white cell counts. The packed cell volume (P.C.V.) was calculated by a computer attached to the counter, from measurements of the mean cell volume (M.C.V.) and red cell count.

Blood was diluted for counting purposes using a Coulter Dual Diluter. The manufacturer's instructions were followed with regard to the dilution of blood, lysis of red cells for white cell counts, setting of the machine and application of corrections for coincident counting.

The counter was frequently checked using standard

blood (Coulter 4C) or blood for which haematological determinations had also been made on another counter or by hand.

In Nigeria, haematological parameters were measured by standard laboratory means. White and red blood cell counts were performed using a Neubauer haemocytometer after dilution with appropriate diluting fluids (Dacie & Lewis, 1966).

Packed cell volumes (P.C.V.s) were determined by the micro-haematocrit method using plain glass capillary tubes and a haematocrit centrifuge (Hawksley and Sons, Ltd., Lancing, Sussex). The P.C.V. was read off on the special scale provided (Hawksley Reader).

Differential white blood cell counts were made on thin smears stained with Giemsa. Two hundred leucocytes were differentiated into lymphocytes, neutrophils, monocytes and eosinophils. Basophils were so infrequently encountered that they were not recorded. Neutrophils were not routinely categorised into immature and segmented forms. Counts were made by the battlement method (MacGregor, Richards & Loh, 1940, cited by Schalm, Jain & Carroll, 1975).

Absolute counts were derived from the differential percentages and white cell counts by multiplication.

Eosinophils were also counted directly. Blood was diluted with Dunger's diluting fluid and eosinophils were counted in a Fuchs-Rosenthal chamber (Dacie & Lewis, 1966).



e) Plasma cortisol determination

The method was that described by Mattingly (1962) and recommended by a Medical Research Council Working Party (1971).

Plasma was prepared within an hour of collection of whole blood using either heparin or E.D.T.A. as anticoagulants. Where possible, cortisol assays were conducted on fresh plasma, but if necessary plasma was stored in a refrigerator at  $+4^{\circ}\text{C}$  for up to 72 hours.

Corticosteroids were extracted from plasma into methylene dichloride ('Dichloromethane' F.D.P.C., B.D.H. Ltd., Poole, Dorset) and then from that into a concentrated sulphuric acid/ethanol mixture ('Fluorescent reagent' F.D.P.C., B.D.H. Ltd.). The fluorescence of the resulting solution was measured at 530 nms. in a fluorimeter ('Eel 244', Evans Electroselenium Ltd., Halstead, Essex).

Together with plasma, a blank, a cortisol standard and a proprietary standard serum ('Seronorm', B.D.H. Ltd) were also run in each test batch.

The method measures both cortisol and corticosterone, but not B-methasone, if this is present in plasma (Mattingly, 1962). Since in bovine blood the concentration of cortisol exceeds that of corticosterone (Edwards, Hardy & Malinowska, 1975) the results will be expressed as plasma cortisol concentrations.

f) Fluorescence microscopy

Three types of fluorescence microscope were used.

In Edinburgh, a Leitz 'Ortholux' was utilised for the examination both of acridine orange stained smears and of indirect fluorescent antibody (I.F.A.) test slides. The settings were the same for both examinations and were as follows. Incident (Ploem, 1967) blue-violet excitation was provided by a mercury vapour lamp (HB 200) with BG 12 and BG 38 primary filters, a K 530 secondary (barrier) filter and turret setting no. 3.

At Vom, acridine orange stained smears were examined by transmitted blue-violet excitation on a Carl Zeiss 'Universal' microscope, with a mercury vapour HB 200 source, appropriate primary and secondary filters and utilising a high transmission, bright field condenser. I.F.A. test slides were examined on a Vickers M15c microscope by transmitted blue-violet excitation from a tungsten halogen 100w. source, with Baltzer and BG 38 2 mm. primary filters and a yellow secondary filter, using a dark-field condenser.

All specimens were viewed unmounted under non-fluorescent immersion oil with either x40, x50 or x54 oil immersion objectives suitable for fluorescent work.

g) The Indirect Fluorescent Antibody (I.F.A.) Test

A standard method was adopted and used for all Babesia spp. tested. This method was similar to that of Ross & Löhr (1968) for B. bigemina and that of Latif (1972) for B. divergens.

### Antigen preparation and storage

Blood with a parasitaemia of at least 10% of the appropriate Babesia spp. was collected from the donor animal using E.D.T.A. as anticoagulant. The donor blood was free of other patent haemoparasites. Plasma was separated from the red cells by centrifugation and then discarded. The red cells were then washed three times in phosphate buffered saline pH 7.2 (P.B.S. - see Appendix 1) by centrifugation and re-suspension in buffer. After the third wash the red cells were re-suspended in buffer to the original blood volume.

Blood smears were prepared for antigen on clean, grease free slides, using another slide to make smears which covered the width and most of the length of the antigen slide. Smears were made of even thickness and were slightly thicker than normal thin blood smears. The antigen smears were allowed to dry and were then wrapped, smear outwards in tissue paper and tin-foil. Packages of four slides were labelled and finally hermetically sealed in plastic bags to which a few crystals of silica gel had been added.

The antigen was immediately stored in either insulated cabinets containing solid carbon dioxide or in an electric freezer (in Vom) at  $-20^{\circ}\text{C}$ .

### Serum preparation and storage

Blood was collected into plain 'Vacutainers', incubated for one hour at  $37^{\circ}\text{C}$  and stored overnight at  $+4^{\circ}\text{C}$ .

Sera were then separated, centrifuged and stored without preservative in glass bijoux bottles at  $-20^{\circ}\text{C}$ .

A bank of reference sera was established. Negative serum was collected from an animal known not to have been infected with Babesia spp. Positive serum was collected from an animal after recovery from laboratory infection with a known Babesia spp.

Reference positive and negative sera were dispensed in 0.15 ml. aliquots in plastic 'polypots' (Luckham, Burgess Hill, Sussex) and stored at  $-20^{\circ}\text{C}$ .

A commercial fluorescein conjugated anti-bovine globulin (Nordic Pharmaceuticals and Diagnostics, Tilburg, Holland) was bought as a freeze-dried powder. It was made up to 2 mls. with distilled water following the manufacturer's instructions. This stock solution was diluted ten-fold with isotonic saline, then dispensed in aliquots and stored like the reference sera.

This conjugate is claimed by the manufacturer to be a specific anti-IgG serum. This was confirmed in our own laboratory by electrophoresis against whole bovine serum and pure bovine IgG.

#### Conduct of the test

On the day of a test, sufficient antigen slides, reference sera and conjugate were removed from cold storage. After the test, any unused reagents were discarded. Antigen slides were allowed to slowly equilibrate to room temperature, by placing them firstly in a refrigerator

at  $+4^{\circ}\text{C}$  for one hour and then leaving them wrapped at room temperature for one hour.

Slides were then unwrapped, identified with a diamond pen and fixed in acetone for five minutes. After fixing, the slides were drained, allowed to dry and then cells were delineated on the smears by red nail-varnish.

In titrations, test sera were diluted to a base dilution of  $1/40$ , then doubling (in the case of B. divergens) or four-fold (in the case of B. bigemina) dilutions were made with P.B.S. pH 7.2 in standard W.H.O. agglutination trays. Dilutions of serum were applied in sequence to the cells on the antigen smears. For screening purposes a single  $1/40$  dilution of each serum was tested.

In addition to the test sera, each antigen slide contained a negative serum and a P.B.S. control cell. A positive serum control cell was also added for screening tests.

Slides, with sera applied, were incubated at room temperature in a moist chamber for thirty minutes. Sera were then washed off with P.B.S. and subjected to two five-minute washes in P.B.S. The washed slides were allowed to dry thoroughly at room temperature.

Aliquots of thawed conjugate at 1 in 10 dilution were further diluted ten-fold in P.B.S. for the test. A 1 in 100 dilution of conjugate was applied to cells on the dry antigen smears and the slides were incubated for a further thirty minutes in a moist chamber. The slides

were finally washed as described above, air-dried and immediately examined unmounted on a fluorescence microscope. The end-point in titrations was taken as the highest dilution to give specific fluorescence.

As a general rule, ten cells were painted on to the antigen slides, so that in titrations in which there were two control cells, this left eight cells for the test sera. With high-titre serum the end-point was roughly determined by preliminary tests and an appropriate range of dilutions was used in the definitive test.

All sera were simultaneously screened or titrated in duplicate and the tests were read blind. Serial samples from any one animal were titrated on the same day.

Where duplicate end-points differed by one dilution interval the lower end-point was accepted as the titre. In instances where the disparity between duplicates was greater than one dilution interval, the test was repeated.

#### (iv) Techniques

##### a) Inoculation

Cattle were infected by the intravenous route of inoculation.

If fresh blood was used, this was inoculated immediately after collection from donor animals into E.D.T.A. 'Vacutainers'. Blood was transferred from the 'Vacutainer' to a syringe fitted with a 18g. x 1½" needle for inoculation.

Stabilates were removed from cold storage, quickly

thawed in cold water and immediately inoculated as described above.

b) Cryopreservation

B. divergens

Freshly collected parasitised blood, for which the parasitaemia and red cell count were known, was distributed in 1 ml. aliquots in glass vials and glycerol was added to a final concentration of 7.5%. The vials were heat-sealed and immediately placed in insulated cabinets containing solid carbon dioxide at  $-79^{\circ}\text{C}$ .

B. bigemina

Freshly collected parasitised blood, for which the parasitaemia and red cell count were known, was distributed in aliquots in screw-top borosilicate glass tubes. Di-methyl sulphoxide was added as cryoprotectant in the ratio of 1 part D.M.S.O. to 10 parts of blood (a final concentration of 9.1%). The vials were immediately placed in liquid nitrogen at  $-196^{\circ}\text{C}$ , and either kept at that temperature or at  $-79^{\circ}\text{C}$  in an electric low temperature freezer (Revco Inc.).

c) Splenectomy

Cattle were splenectomised between six weeks and six months of age. Before the operation calves were starved for two days and deprived of water for one day.

The anaesthesia employed in Edinburgh was different from that used in Vom, but the surgical technique was the same in both places.



In Edinburgh, anaesthesia was induced by administration of a mixture of halothane ('Fluothane', I.C.I., Macclesfield, Cheshire), nitrous oxide and oxygen through a face mask. Calves were then intubated and anaesthesia was maintained with the same mixture of gases administered through an endotracheal tube.

At Vom, pentobarbitone sodium ('Nembutal') was used to induce and maintain anaesthesia. A 'knock-down' dose was administered into the jugular vein by a syringe and needle which were then taped to the neck with the needle in the lumen of the vein. Anaesthesia was maintained by further injections of small volumes of pentobarbitone. Though somewhat unconventional this system worked very well. One calf died due to adverse reaction to what should have been a safe dose of pentobarbitone. Eight calves were successfully anaesthetised for splenectomy by this means.

The surgical technique, demonstrated to us by Dr. Wilde, was as described by Gates, (1953).

Calves were placed on their right side and the left flank shaved. With full aseptic precautions a left flank incision was made parallel to and a few centimetres posterior to the last rib. The incision was about 20 cms. long, stretching from just ventral to the transverse processes of the lumbar vertebrae to the mid-flank.

The peritoneal investments of the spleen were severed manually, until only the splenic hilus attached the spleen to the viscera. At this stage the spleen could be exter-

iorised. The hilus was transfixed and ligated with 0/2 chromic catgut and the spleen was removed. The abdominal muscles were joined by continuous suture with 0/2 chromic catgut and the skin incision was closed with mattress sutures of nylon or chromic catgut. A streptomycin/penicillin ointment ('Streptopen') was squeezed into the incision, and a fly-repellent powder ('Negasunt', Bayer U.K. Ltd., Bury St. Edmunds, Suffolk) was puffed on the wound.

d) Staining methods

Giemsa

Thin blood smears were fixed in technical methanol and then stained in staining baths with Giemsa. A 10% dilution of stock Giemsa solution was made up in phosphate buffer pH 7.2 (see Appendix 1) and slides were stained for 30 minutes or one hour. Stained smears were briefly rinsed in the same buffer pH 7.2, and allowed to drain and dry.

Giemsa stock solution was either a proprietary brand (Gurr, Searle Diagnostic, High Wycombe, England) or was made up in the laboratory from Giemsa crystals, glycerol and methanol (Dacie & Lewis, 1966).

Home made stock solution was allowed to mature for several weeks before it was used.

Whichever stock solution was used, it was filtered before use. Staining solution was made freshly each day, and used within an hour or two of preparation.

Acridine orange

A stock solution of 0.1% w./v. of acridine orange (Gurr, Searle Diagnostic, High Wycombe, England) was made up with distilled water. A few crystals of sodium azide were added to each 100 mls. of stock solution to prevent fungal contamination. The stock solution was occasionally filtered, but otherwise could be kept for several months.

The staining solution was made up by adding one part stock solution to nine parts of McIlvane's buffer pH 6.4 (see Appendix 1), making a final solution of 0.01% acridine orange. This staining solution could be used for several days.

Thin blood smears were fixed in technical methanol for two minutes. Slides were then thoroughly air-dried. The dry, fixed smears were then stained for one minute and rinsed in McIlvane's buffer for five minutes. The exact times for staining and rinsing were occasionally adjusted to give optimum staining quality. Smears were examined, unmounted, by fluorescence microscopy.

e) Brain biopsy and brain crush smears

These techniques were employed in Nigeria where B. bovis was encountered.

The biopsy technique of Johnston & Callow (1963) was followed with slight differences. The site of the operation is shown in Plate 3. Calves were held by hand and the biopsy site was infiltrated with local anaesthetic. Having made a small skin incision, the cranium was pene-

Plate 3

Site of brain biopsy operation





trated using an ordinary twist drill and a conventional 3/32" bit. An 18g. x 1½" needle attached to a 20 mls. plastic syringe was used to obtain the biopsy material. It was found that movements of the needle and syringe plunger were critical if biopsy material was to be obtained in the needle. The following procedure was found to work well:

- (i) the needle was introduced into the brain,
- (ii) the syringe was attached and the plunger withdrawn to 5 mls.,
- (iii) the plunger was released,
- (iv) syringe and needle were withdrawn,
- (v) the needle and syringe were separated and the needle contents were expelled on to a slide.

If this series of actions was not carried out the biopsy sample was sucked into the syringe where it was inaccessible.

Brain crush smears were prepared from biopsy and post-mortem material by the method of Purchase (1945) employing Leeflang's modification (Leeflang, 1972). Smears were stained by Giemsa's method.

### C. Ticks

Engorged female Boophilus spp. ticks were identified by examination of the mouthparts under a dissecting microscope, following the key given by Hoogstraal (1956).

They were weighed and placed individually in labelled

glass tubes stoppered with a cotton wool and muslin plug. The tubes were kept in a humidity cabinet in which air was circulated by a fan and the humidity maintained at 80-85% over a saturated solution of sodium carbonate. The temperature inside the cabinet was not controlled but was monitored by means of a maximum-minimum mercury thermometer.

The ticks were examined frequently and a record was kept of when eggs were laid and when they hatched. Egg batches were weighed fifteen days after the first eggs were laid, by which time laying had ceased.

Larval ticks were scattered over the backs of calves held in pens in the tick-feeding shed (described under 'Feeding, management and housing of cattle'). Engorged ticks were collected each morning from the floor of the pens.



## EXPERIMENTS ON RODENTS

### A. Babesia rodhaini in mice

#### Introduction

Babesia rodhaini was first discovered parasiting the tree rat Thamnomys surdaster surdaster in the then-named Belgian Congo (Berghe, Vincke, Chardome & Bulke, 1950). Infections were readily established in mice and the course of infection and the morphology of the parasite have been described by the original discoverers and by many others since. When first isolated, inoculation into mice produced mild infections with peak parasitaemias of 10-20% and only one death in 17 mice (Berghe, Vincke, Chardome & Bulke, 1950) but continuous passage quickly exalted the virulence of the organism. Rodhain (1950) reported parasitaemias of over 80% and about 25% mortality, and Colas-Belcour & Vervent (1953) obtained recovered mice, some of which were latently infected for months, but when Paget, Alcock & Ryley (1962) studied the parasite they found it to be highly pathogenic, killing virtually all infected mice.

Aeschlimann & Suter (1965) described high mortality rates in mice and found that the prepatent period was related to the number of parasites inoculated. BünGENER (1967) and Overdulse & Antonisse (1970) investigated this relationship in greater detail. They established that the prepatent period and the time to death were inversely

proportional to the logarithm of the number of parasites inoculated. Overdulse & Antonisse (1970) derived the generation time for their strain of B. rodhaini and used the titration principle to assess the efficiency of cryopreservation. Roberts & Tracey-Patte (1975a) employed titrations in order to assess the number of parasites which survived treatment with immune serum.

Whilst it seemed unlikely that B. rodhaini in mice would prove suitable for studies of chronic parasitaemia, titration experiments were performed in order to characterise the strain and in an attempt to produce natural survivors. Observations on infections in mice, made at other times, are also presented.

### Experiments

#### a) Characteristics of B. rodhaini infection in mice

##### Materials and methods

The data were obtained from mice infected during the passage maintenance of B. rodhaini and from mice specifically infected in various experiments.

##### Results

A typical parasitaemia curve is shown in Fig. 3. Parasitaemia rose very rapidly from sub-patent to high levels at which time death occurred. Death was preceded by one or two days of illness (rarely more), in which mice were depressed, anaemic, had 'staring' coats and exhibited haemoglobinuria. A few mice survived a milder infection in which urine was discoloured green, but not red, and a



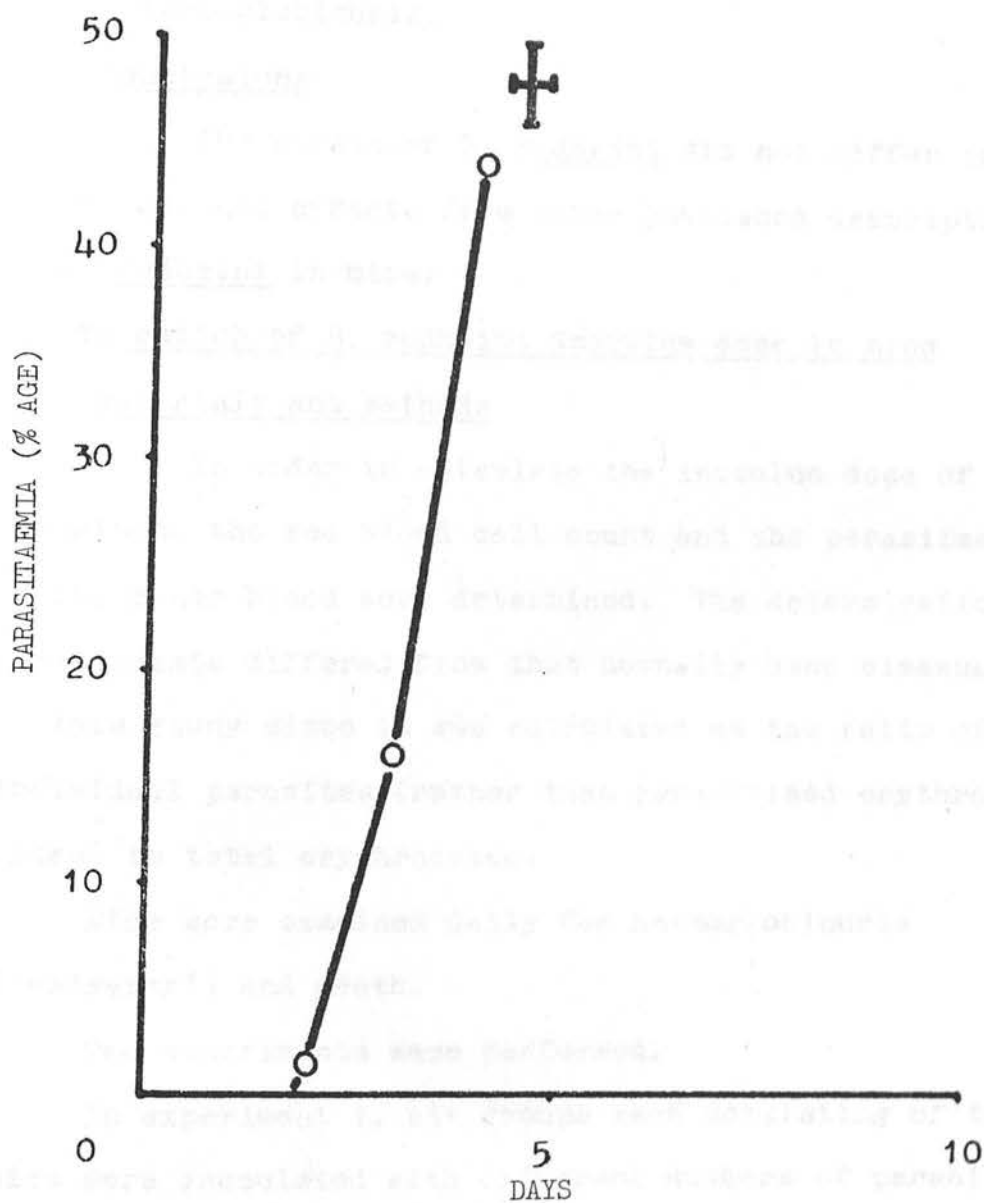


Fig. 3

A typical parasitaemia curve of

B. rodhaini in a mouse



few survived without symptoms of illness.

Of a total of 150 mice which were infected with B. rodhaini infected mouse blood, 94.6% died and 95.2% showed haemoglobinuria.

### Conclusions

The strain of B. rodhaini did not differ in its morphology and effects from other published descriptions of B. rodhaini in mice.

### b) Titration of B. rodhaini inoculum dose in mice

#### Materials and methods

In order to calculate the inoculum dose of parasites, the red blood cell count and the parasitaemia of the donor blood were determined. The determination of parasitaemia differed from that normally used elsewhere in this study since it was calculated as the ratio of individual parasites (rather than parasitised erythrocytes) to total erythrocytes.

Mice were examined daily for haemoglobinuria ('redwater') and death.

Two experiments were performed.

In experiment 1, six groups each consisting of ten mice were inoculated with different numbers of parasites/mouse ranging from  $1.34 \times 10^8$  -  $1.34 \times 10^3$  in tenfold increments. In addition a control group was inoculated with  $1.34 \times 10^8$  parasites/mouse - this inoculum was subjected to the physical treatment associated with the dilution procedure and the group was the last to be

inoculated.

In experiment 2, six groups of mice were inoculated with  $1.77 \times 10^8$ ,  $1.77 \times 10^7$ ,  $1.77 \times 10^3$ ,  $1.77 \times 10^2$ ,  $1.77 \times 10^1$  and  $1.77 \times 10^0$  parasites/mouse respectively. There were twelve mice in each group with the exception of the first group which consisted of ten and the last which consisted of six.

Forty-six days after inoculation all the survivors were challenged with heavily parasitised B. rodhaini infected mouse blood.

### Results

#### Experiment 1

The mortality rate for each inoculum dosage is shown in Table 1. Taking the two most divergent figures for groups infected with  $1.34 \times 10^6$  and  $1.34 \times 10^5$  parasites, by the chi-square test there is no significant difference between the proportions of survivors in these two groups ( $p = > 0.05$ ).

The overall mortality rate was 63/70 or 90%. Haemoglobinuria was observed in all mice which died and in 4/7 of the survivors. Of the three surviving mice in which haemoglobinuria was not observed, two were clinically ill following infection.

The reactions of the two groups inoculated with  $1.34 \times 10^8$  parasites were almost identical in all parameters. This shows that the infective dose of parasites was not reduced by the physical effects of the dilution

Table 1

The mortality rates in mice inoculated with different numbers of Babesia rodhaini (Experiment 1)

No. of <u>B. rodhaini</u> inoculated	Died	Survived
$1.34 \times 10^8$	9	1
$1.34 \times 10^8$	10	0
$1.34 \times 10^7$	9	1
$1.34 \times 10^6$	10	0
$1.34 \times 10^5$	7	3
$1.34 \times 10^4$	9	1
$1.34 \times 10^3$	9	1

Table 2

The mortality rates in mice inoculated with different numbers of Babesia rodhaini (Experiment 2)

No. of <u>B. rodhaini</u> inoculated	Died	Survived
$1.77 \times 10^8$	10	0
$1.77 \times 10^7$	11	1
$1.77 \times 10^3$	12	0
$1.77 \times 10^2$	4	8
$1.77 \times 10^1$	0	12
$1.77 \times 10^0$	0	6



procedure, or by the delay which this entailed between the collection of the infected blood and its inoculation.

The time to the onset of haemoglobinuria and the time to death varied inversely with the  $\log_{10}$  inoculum dosage. The relationship is illustrated in Fig. 4 where median values for each group are plotted. The data were subjected to regression analysis and the lines of best fit determined by the method of least squares. The linear correlation between inoculum dosage and time to death is highly significant (correlation coefficient = -0.99,  $p = < 0.001$ ). The regression curves for time to death and time to haemoglobinuria are each highly significant ( $p = < 0.001$ ) and have gradients of -2.24 and -2.0 respectively. The regression curves differ significantly in constant ( $p = < 0.01$ ) but not in slope ( $p = > 0.05$ ).

The rate of multiplication of B. rodhaini can be calculated from the formula given by Overdulve & Antonisse (1970) i.e.

$$\lambda = \bar{b} \log_{10} 2$$

where  $\lambda$  is the time between successive binary fissions, and  $\bar{b}$  is the gradient of the regression for time to death or prepatent period. Taking the average of the two gradients calculated above, substitution in the formula gives a generation time of 15.3 hours.

### Experiment 2

The mortality rate at each inoculum dosage is shown in Table 2. The mortalities for the groups must be

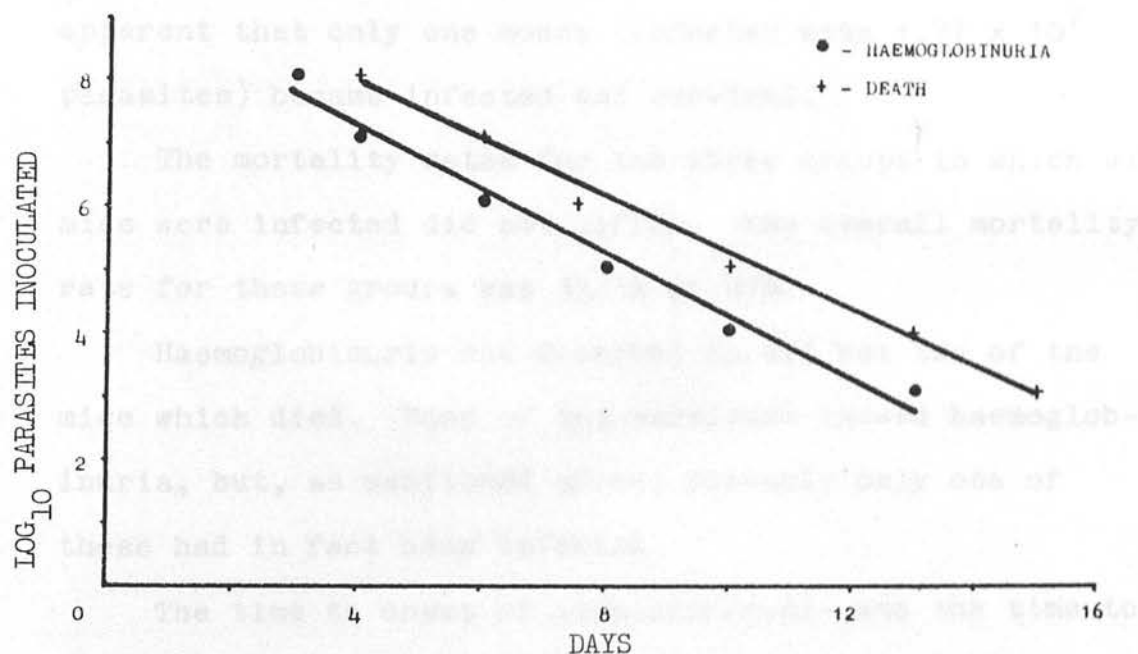


Fig. 4

The titration of B. rodhaini in mice

(Experiment 1)



considered in conjunction with the results of the challenge of survivors which are tabulated in Table 3. When challenged, virtually all the so-called survivors proved fully susceptible. In other words, they had not been truly infected by the inoculation of very low numbers of B. rodhaini. Taking the acquisition of immunity to subsequent challenge as a criterion of infection, it is apparent that only one mouse (infected with  $1.77 \times 10^7$  parasites) became infected and survived.

The mortality rates for the three groups in which all mice were infected did not differ. The overall mortality rate for these groups was 33/34 or 97%.

Haemoglobinuria was observed in all but two of the mice which died. None of the survivors showed haemoglobinuria, but, as mentioned above, probably only one of these had in fact been infected.

The time to onset of haemoglobinuria and the time to death varied inversely with the  $\log_{10}$  inoculum dosage. The relationship is illustrated in Fig. 5 where median values for each group are shown, and the median value for the group inoculated with  $1.77 \times 10^2$  parasites is also included. The data were subjected to regression analysis and the lines of best fit determined by the method of least squares. The linear correlation between inoculum dosage and time to death is significant (correlation coefficient = -0.96,  $p = < 0.05$ ) as is that between inoculum dosage and time to haemoglobinuria (correlation



Table 3

Results of challenge of mice surviving from Babesia  
rodhaini titration (Experiment 2)

Titrated inoculum	No. of survivors	Results of challenge	Median time to death at challenge (days)
$1.77 \times 10^7$	1	Survived	-
$1.77 \times 10^2$	8	8/8 Died	6
$1.77 \times 10^1$	12	12/12 Died	6
$1.77 \times 10^0$	6	3/6* Died	7
Challenge controls		6/6 Died	6

\*(2/3 of challenge survivors showed haemoglobinuria)

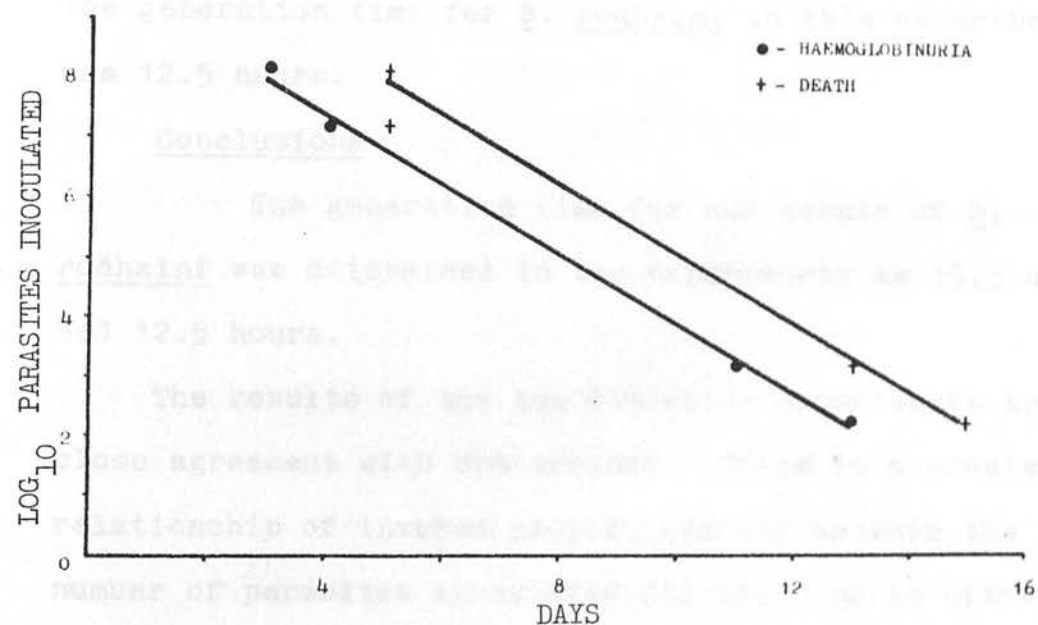


Fig. 5

The titration of B. rodhaini in mice

(Experiment 2)

coefficient = -0.98,  $p = < 0.05$ ).

The regression curves for time to death and time to haemoglobinuria are each highly significant ( $p = < 0.001$ ) and have gradients of -1.73 and -1.72 respectively. The regression curves differ significantly in constant but not in slope ( $p = < 0.05$  and  $p = > 0.05$ , respectively).

Applying the formula of Overdulve & Antonisse (1970), the generation time for B. rodhaini in this experiment was 12.5 hours.

### Conclusions

The generation time for our strain of B. rodhaini was determined in two experiments as 15.3 hours and 12.5 hours.

The results of the two titration experiments are in close agreement with one another. There is a precise relationship of inverse proportionality between the  $\log_{10}$  number of parasites inoculated and the time to either haemoglobinuria or death. This relationship holds true over most of the range of infective doses down to the threshold of infection, which was determined to be about  $10^2$  parasites. In this experiment 8/12 of mice inoculated with this number of parasites, and all inoculated with fewer parasites, did not become infected and remained susceptible to B. rodhaini at challenge.

The overall mortality rate was high and was not influenced by the inoculum size.



## Discussion

The results of these titrations broadly agree with those of other workers. The determined generation times of 15.3 hours and 12.5 hours were very similar to the 14.5 hours obtained by Overdulse & Antonisse (1970). A  $\log_{10}$  increase in parasitaemia occurred in 46 hours compared with the 34 hours observed by Büngener (1967), and with an increase of parasitaemia by a factor of 5.5 in 24 hours noted by Nowell (1969).

The relationship between 50% survival time and the number of parasites inoculated was found by Roberts & Tracey-Patte (1975a) to hold over a range of inocula from  $10^{1.5}$  to  $10^8$ . This seems inconsistent with their observation that the multiplication rate of the parasite decreased as the inoculum decreased. They reconcile these two facts by reasoning that the effect on multiplication rate will only occur during the latter stages of infection with small doses of parasite. Such a phenomenon would account for the slight deviations from a strictly linear relationship seen at the extremes of the regressions (Figs. 4 and 5), but in general the results of our experiments and those of Overdulse & Antonisse (1970) suggest that the parasite multiplication rate is not a function of inoculum size. Overdulse & Antonisse (1970) obtained a very few surviving mice at the lower end of their range of inoculum doses. Most mice which survived were probably not infected since only one was proved to

harbour a chronic parasitaemia. This is in accordance with our own results i.e., that the infection of mice with B. rodhaini is generally speaking an 'all or none' phenomenon in which the mortality rate is not influenced by the inoculum size. This has the important practical corollary that naturally recovered mice which are chronically infected, cannot be obtained in reasonable numbers by varying the number of parasites inoculated.

It is also interesting to note that the period of illness, as denoted by the duration of haemoglobinuria, was not affected by varying the inoculum dose, although this altered the time scale of the infection as a whole. There was no indication in these experiments, in spite of times to 'redwater' of as much as 13 days, of a defensive host response modifying the course of disease. Similarly, Overdulse & Antonisse (1970) found that the duration of parasitaemia was not affected by the inoculum size. In view of the findings of Roberts & Tracey-Patte (1975a) which showed that small doses of passively administered antisera reduced the multiplication rate of parasites, it appears that even mice infected with low numbers of B. rodhaini do not produce antibody before death. Considered in conjunction with the observation of Cox & Turner (1970) that mice are capable of producing antibody within six days of infection by a piroplasm (B. microti), it seems likely that either B. rodhaini can block the host defence responses, or that there is a threshold parasitaemia which

is quite high and below which there is insufficient antigenic stimulus to provoke a host response.

The high mortality rates obtained with B. rodhaini infections of mice render this system unsuitable for studies of chronic infection after natural cure, but make it particularly suitable for chemotherapeutic trials (Beveridge, 1953). Perhaps because of this, a great deal of attention has been focussed on this host-parasite system as a model for those Babesia spp. of economic importance. It is worth emphasising however, that in many respects B. rodhaini is far from ideal for this purpose. The vector of B. rodhaini is not known, and in spite of efforts to transmit the parasite biologically (Young, 1970) it must as yet be transmitted by needle passage. This has enhanced the virulence of the parasite to such an extent that natural recoveries are rare. In contrast, the Babesia spp. of veterinary importance are characterised by vector infection from which the majority of animals recover to become chronically infected.

B. Babesia rodhaini in rats

Introduction

Beveridge (1953) first adapted B. rodhaini to white rats and described the infection produced. Since then numerous studies have been reported (Matson, 1964; Phillips, 1968; Nowell, 1969; Young, 1970 and others). This work shows that B. rodhaini is not as virulent in rats as in mice and that a proportion of infected rats recover naturally. This proportion depends on such factors as the inoculum dose, the age of the rat and whether the parasite is maintained in mice or rats. These factors also affect the characteristics of the parasitaemia produced. Large numbers of parasites inoculated into young rats cause high parasitaemias and short prepatent periods, whereas lower inoculum doses in mature rats are followed by low parasitaemias and long prepatent periods (Nowell, 1969). If the rat-adapted strain is maintained in mice, then passage to rats results in maximum parasitaemias of 20-30% followed by recovery, but infection of rats with the rat-adapted strain maintained in rats causes higher parasitaemias (60-70%) and some deaths (McHardy, 1967).

Various authors have shown that recovered rats are chronically infected. By splenectomy and subinoculation, Phillips (1969a) found that 50% of recovered rats still harboured parasites three months after infection and that a few rats were still infected after one year. He found that splenectomy was slightly better than the subinoc-

ulation of blood into mice for revealing latent infections. Young (1970) found little difference in the efficiency of the two techniques. In his experiments all rats were latently infected at 3 months and some were still infected at 12 months after inoculation with parasites.

In the light of evidence that primary infection of rats with B. rodhaini is succeeded by a period of chronic latent infection, a series of studies was commenced with the intention of using this model system to investigate chronic parasitaemias and relapse provocation.

The work began with attempts to derive a rat-adapted strain of B. rodhaini. These are described in section a) together with the characteristics of infections. The studies continued with investigations into the duration of infection as determined by splenectomy and subinoculation (section b). In section c) the immunity of recovered rats to challenge is described.

## Experiments

### a) The adaptation of B. rodhaini to rats and the characteristics of infection therein

#### Materials and methods

Blood from two mice with high parasitaemias of B. rodhaini (30% and 40%) was pooled and 0.5 ml. was inoculated intraperitoneally into each of two non-splenectomised rats. From these, a series of passages was carried out in groups of rats. Blood was successively

subinoculated from the rat with the highest parasitaemia in each passage group.

### Results

Both rats which were inoculated with infected mouse blood developed patent parasitaemias. After five passages a rat-adapted strain was derived which consistently produced infections with high parasitaemias. Rats with parasitaemias in excess of about 30% became dull and depressed, the ears were pale and haemoglobinuria was usually evident. Most rats recovered without treatment. The mortality rate varied during the course of this study in relation to the number of times the parasite was passaged. This aspect is dealt with in greater detail later.

The typical course of parasitaemia is illustrated in Fig. 6. It is characterised by a rapid multiplication phase, a crisis phase and a resolution phase when the parasitaemia drops to sub-patent levels within a day or two of maximum parasitaemia.

The morphology of the parasite during rising parasitaemia is shown in Plate 4. Occasionally four parasites which were joined centrally and arrayed in a cross shape (the so-called 'Maltese Cross') were seen. Well defined morphological changes in the parasite were sometimes observed, associated with the resolution phase of parasitaemia. Organisms were smaller, spherical and 'glossy' after Giemsa staining, an appearance which appositely came to be known as the 'droplet' form (Plate 5a). This



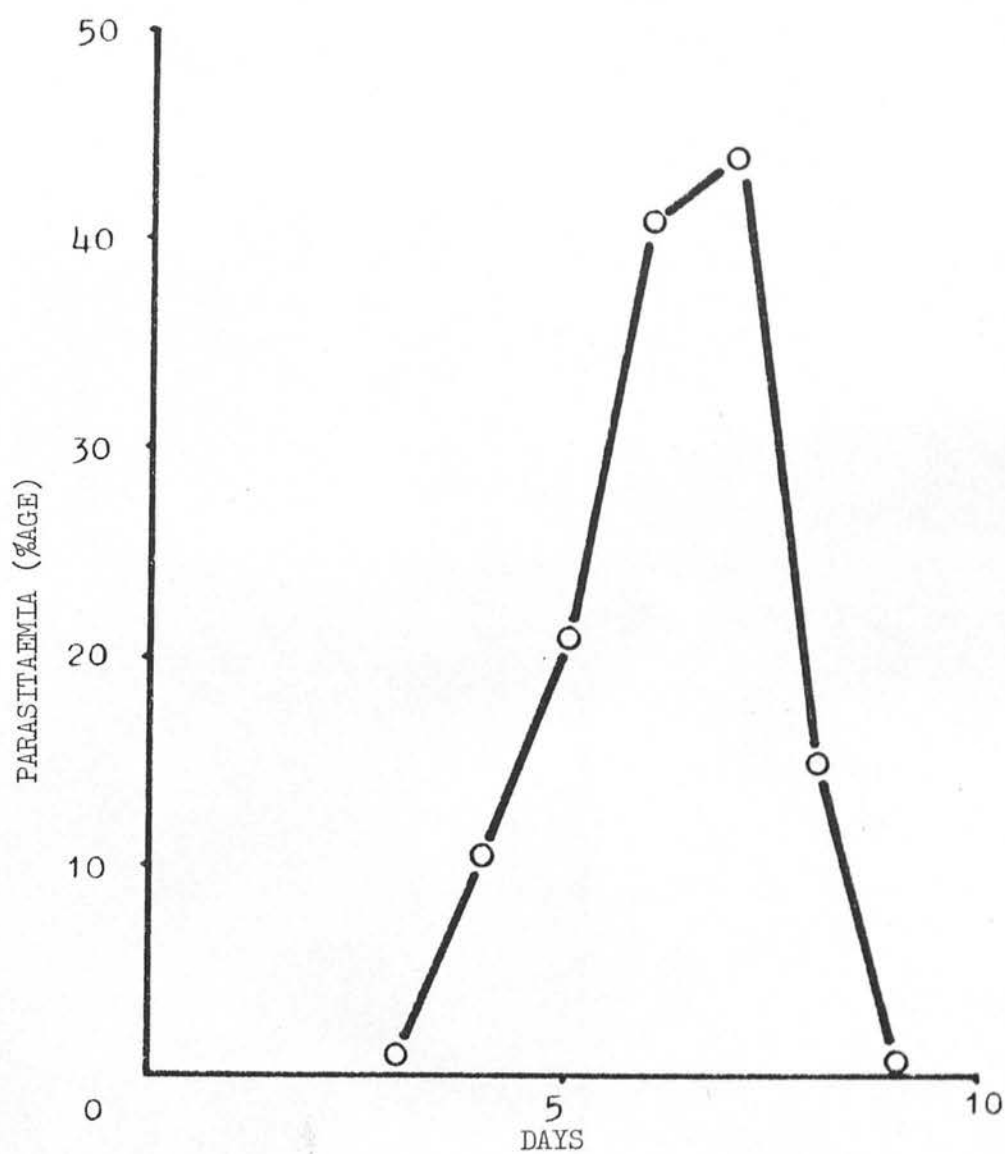


Fig. 6

A typical parasitaemia curve of B. rodhaini  
in a rat

Plate 4

Babesia rodhaini in the rat - a  
rising parasitaemia

Plate 5

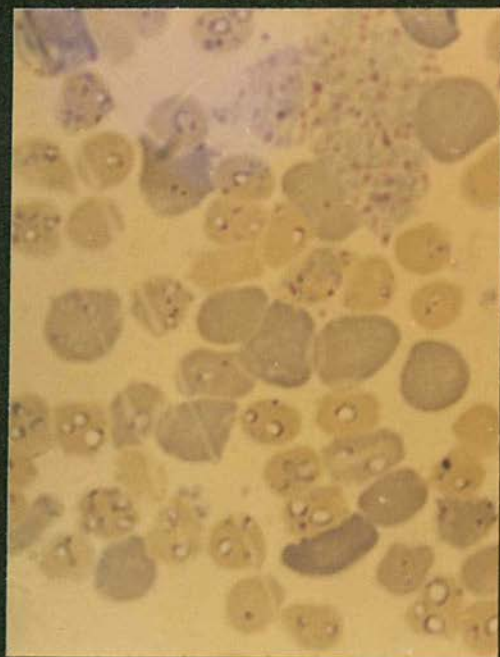
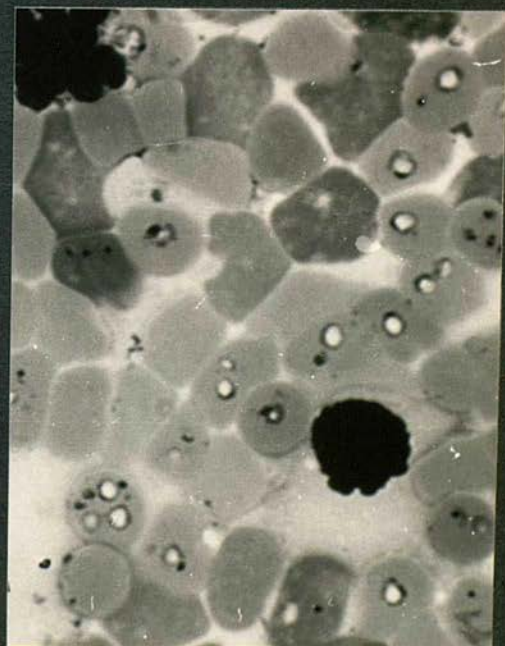
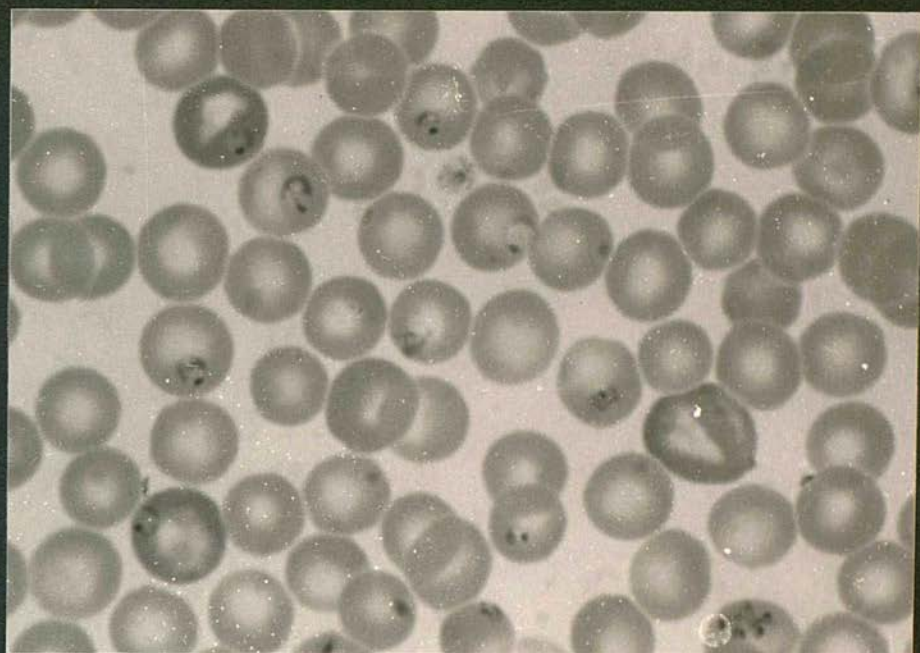
Babesia rodhaini in the rat -  
crisis forms:

a)

'droplet' form at  
natural cure

b)

after drug treatment  
(by courtesy of  
R.G. Philip)



form was transient and was not observed on more than one occasion in any one animal.

### Conclusion

A mouse strain of B. rodhaini was adapted, without difficulty, to rats. It caused moderate parasitaemias, with varying but usually slight degrees of illness from which most rats recovered. When the parasitaemia began to fall abnormal forms of the parasite were conspicuous but transient.

### Discussion

The mouse strain of B. rodhaini proved to be easily adaptable to rats without recourse to splenectomy or immunosuppression. This was not so when the strain was first isolated in 1950 and several attempts to infect rats met with little success, until Beveridge (1953), with some difficulty, finally succeeded in establishing a rat strain.

The parasitaemia curve (Fig. 6) is similar to that presented by Nowell (1969), and in terms of pathogenicity our rat-adapted strain resembled strains previously described in the literature.

The presence of spherical, refractile parasites at crisis in naturally recovering rats, does not seem to have been reported before, although various authors have examined morphological changes in the course of B. rodhaini infections (Aeschlimann & Suter, 1965; Nowell, 1969; Young, 1970). From a comparison with the appearance

of parasites after drug treatment, (Plate 5b - Philip, 1975), it would appear that these forms are dead or dying parasites. Their transient presence suggests that they were quickly eliminated from the circulation. What were presumed to be dead parasite remnants were observed by Clark, Wills, Richmond & Allison (1975) in B. microti infections of mice. These remnants were dense pyknotic bodies, rather than large, refractile parasites, and they persisted for some time.

b) The duration of B. rodhaini infection in rats,  
(including an assessment of splenectomy and  
subinoculation as methods of detecting latent  
infections)

(i) The duration of infection as determined by  
splenectomy

#### Materials and methods

Rats which had recovered from infection during the maintenance of B. rodhaini by passage were splenectomised as they were available. The primary parasitaemia in each of these rats exceeded 1% and in most of them it exceeded 20%. Primary parasitaemias were regarded as sub-patent when they fell below 0.1%. None of the rats had a patent parasitaemia when splenectomised. Thin blood smears were examined daily for 28 days after splenectomy.

#### Results

The results are shown in Table 4. Only one of

Table 4

The persistence of B. rodhaini in rats as determined by  
splenectomy

Days since inoculation	Number splenectomised	Number infected
0 - 20	5	1
21 - 40	3	0
41 - 60	5	0
61 - 80	1	0
81 - 100	6	0
101 - 120	4	0
Totals	24	1

Table 5

The persistence of B. rodhaini in rats as determined by  
splenectomy, (detailed data from first five rats in  
Table 4)

Days since inoculation	Days since onset of latency	Result of splenectomy
11	1	+ve
14	?	-ve
15	1	-ve
17	7	-ve
17	8	-ve



twenty-four rats was found to be latently infected. The results for the first group of five rats are analysed further in Table 5. These results suggest that if the failure to relapse after splenectomy is regarded as evidence that a sterile state exists, then these rats had effected a sterile cure within fourteen days of infection, and between one to seven days after the primary parasitaemia became sub-patent.

### Conclusions

Persistent latent infection did not appear to follow primary infection in these rats.

- (ii) The duration of infection as determined by the subinoculation of blood from recovered rats into mice

### Materials and methods

The duration of infection was determined in twelve rats which had recovered from B. rodhaini inoculation at various times during the maintenance of the parasite by passage. All of these rats had suffered primary parasitaemias which exceeded 10%, but none had a patent parasitaemia at the time of subinoculation. The level of patency\* was 0.1%. When the surviving subinoculated mice were challenged with B. rodhaini infected rat blood 60 days after subinoculation, two susceptible mice were included as positive controls. Mice were examined daily for haemoglobinuria and death for 18 days after

\* - see Appendix 2

subinoculation or challenge.

Standard criteria for deciding the result of the test were adopted, and were applied to this and all subsequent subinoculations. This was necessary because, as previously described results show, not all mice infected with B. rodhaini show haemoglobinuria or die. The criteria were as follows. A test was regarded as positive and the rat was considered to be infected if at least one of the pair of subinoculated mice showed haemoglobinuria, and if mice deemed infected by this standard resisted challenge. A test was regarded as negative if neither of two mice subinoculated with blood showed haemoglobinuria, and at least one of these showed haemoglobinuria after challenge. (In practice, both mice usually showed haemoglobinuria and died after challenge).

### Results

Both positive control mice showed haemoglobinuria and died after inoculation with parasitaemic blood. The results for the subinoculations from the twelve recovered rats are shown in Table 6. Only two of the twelve rats were found to be infected. Table 7 illustrates data from the first five rats of Table 6 in greater detail. If the failure to infect either one of a pair of mice is regarded as evidence that a sterile state exists, then it appears that rats effected a sterile cure within 25 days of infection and between 4 and 14 days after the primary parasitaemia became sub-patent.

Table 6

The persistence of B. rodhaini in rats as determined by  
the subinoculation of blood into mice

Days since inoculation	Number of rats tested	Number of rats infected
0 - 20	2	2
21 - 40	3	0
41 - 60	1	0
61 - 80	3	0
81 - 100	3	0
Totals	12	2

Table 7

The persistence of B. rodhaini in rats as determined by  
the subinoculation of blood into mice  
(detailed data from first five rats in Table 6)

Days since inoculation	Days since onset of latency	Result of subinoculation
11	1	+ve
13	4	+ve
25	14	-ve
40	31	-ve
40	31	-ve

### Conclusion

This experiment confirmed the somewhat surprising absence of persistent latent infections in recovered rats.

#### (iii) A comparison of splenectomy and subinoculation as means of detecting latent infection in rats

This was carried out to extend the findings on the duration of latent B. rodhaini infection revealed by splenectomy and subinoculation and to compare the sensitivity of these detection methods.

### Materials and methods

Twenty-one rats were inoculated with  $6.3 \times 10^7$  parasitised red blood cells. Thin blood smears from them were examined from day six post-infection\*. The rats were divided into five groups which were tested for latent infection at 10, 12, 14, 16 and 18 days post-infection. In each group some rats were splenectomised and the others had blood subinoculated from them into mice.

Thin blood smears from splenectomised rats were examined daily for 28 days. Subinoculated mice were examined daily for 25 days. Surviving mice were challenged with B. rodhaini infected rat blood.

Primary parasitaemias in the rats were regarded as sub-patent when the parasitaemia fell below 0.1%.

### Results

One rat died on day 2. The other 20 rats all

\* - see Appendix 2

became infected and showed high parasitaemias. The mean observed maximum parasitaemia was 35.2% (range 6% - 66%). Two rats died. The development of parasitaemias was - with few exceptions - very uniform. Observed maximum parasitaemia occurred in all but three rats on day 6. Parasitaemias in almost all of the rats were sub-patent by day 9.

The results of the experiment are summarised in Table 8.

One splenectomised rat suffered a non-fatal relapse parasitaemia which became patent 14 days after splenectomy, reached a peak of 58% six days later and became sub-patent in a further five days.

The proportion of subinoculated mice which became infected, decreased as the length of time between onset of latency and subinoculation increased. The times to death in the mice which became infected are shown in Fig. 7.

### Conclusions

The fact that fewer mice became infected as the time between onset of latency and subinoculation increased, suggests that the threshold dose for infection was being reached. Examination of the time to death in mice which became infected confirms this (Fig. 7). The time increased exponentially as the time between onset of latency and subinoculation increased. If the time to death in subinoculated mice is related to the titration

Table 8

The detection of latent B. rodhaini in rats by  
splenectomy and subinoculation

SPLENECTOMY

Rat no.	Days infected before splenectomy	Days of latency before splenectomy	Result
A2	10	2	+ve
B1	12	4	-ve
B2	12	4	-ve
C1	14	6	-ve
C2	14	6	-ve
D1	16	8	-ve
D2	16	7	-ve
E1	18	5	-ve
E2	18	10	-ve

SUBINOCULATION

Rat no.	Days infected before subinoculation	Days of latency before subinoculation	Result
A3	10	2	+ve
A4	10	2	+ve
B3	12	4	+ve
B4	12	4	+ve
C3	14	7	+ve
C4	14	6	+ve
D3	16	1	+ve
D4	16	9	-ve
E3	18	10	-ve



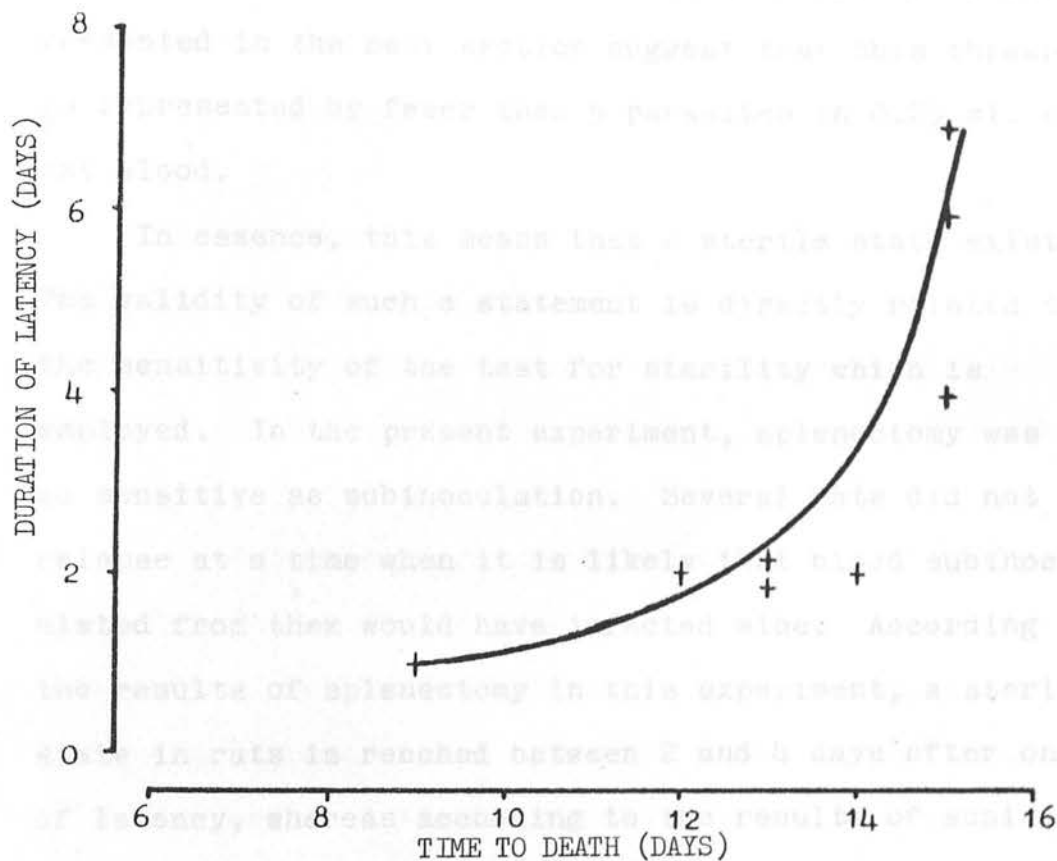


Fig. 7

The time to death in mice, subinoculated with a B. rodhaini infected rat blood, related to the duration of latency in donor rats.

results in Figs. 4 and 5, it can be seen that the  $\log_{10}$  number of parasites in the subinoculum decreased as the period of latency increased. A point is reached where the number of parasites in an inoculum is insufficient to infect mice, and the results of rat to mouse titrations presented in the next section suggest that this threshold is represented by fewer than 5 parasites in 0.25 ml. of rat blood.

In essence, this means that a sterile state exists. The validity of such a statement is directly related to the sensitivity of the test for sterility which is employed. In the present experiment, splenectomy was not as sensitive as subinoculation. Several rats did not relapse at a time when it is likely that blood subinoculated from them would have infected mice. According to the results of splenectomy in this experiment, a sterile state in rats is reached between 2 and 4 days after onset of latency, whereas according to the results of subinoculation, a sterile state is reached 7-9 days after onset of latency.

The results agree with those of previous experiments in which subinoculation appeared to be as sensitive a means of detecting latent infections as splenectomy.

Because of the strict relationship which exists in mice between the  $\log_{10}$  number of parasites inoculated and the time to haemoglobinuria or the time to death, subinoculation provides a means of quantifying levels of

parasitaemia of B. rodhaini in rats, well below those which could be measured by other means.

From the results of these experiments a standard means of testing for latency was adopted, involving subinoculation ten days after the primary parasitaemia became sub-patent.

(iv) The titration of B. rodhaini infected rat blood into mice

This was carried out in order to find out how sensitive the subinoculation method was in detecting B. rodhaini in rat blood, and to determine what relationship existed between inoculum dosage and time to death.

Materials and methods

Infected blood was diluted with non-infected rat blood - rather than with the usual buffered salt solution - in order to simulate different levels of parasitaemia. The volume of inoculated blood was 0.25 ml. for each mouse (as was used in subinoculation experiments). Groups of eight mice were inoculated with  $5.7 \times 10^6$ ,  $5.7 \times 10^5$ ,  $5.7 \times 10^4$ ,  $5.7 \times 10^3$ ,  $5.7 \times 10^2$ ,  $5.7 \times 10^1$  and  $5.7 \times 10^0$  parasites/mouse, and one group was inoculated with non-infected rat blood. This latter group served as susceptible controls when surviving mice were challenged ninety days after subinoculation. Mice were inspected daily for haemoglobinuria and death for twenty days after subinoculation and challenge. Occasional thin blood smears were also examined.

## Results

Of the fifty-six mice inoculated, forty-nine died after showing haemoglobinuria. The mortality rates at each titration interval are shown in Table 9. By chi-square analysis, there is no significant difference between the mortality rates in any of the groups ( $p = > 0.2$ ). Of the seven surviving mice, four exhibited haemoglobinuria and two of the other three were found, by examination of blood smears, to be infected. Only one mouse, inoculated with  $5.7 \times 10^0$  parasites, was not shown to have been infected. All the surviving mice withstood challenge without evidence of haemoglobinuria, though low parasitaemias were observed in three of them. The control mice all showed haemoglobinuria at challenge, and all, except one, died.

Thus all the mice, with the possible exception of one, were infected with B. rodhaini even with an inoculum containing as few as a theoretical 5.7 parasites.

The times to haemoglobinuria and to death were inversely proportional to the  $\log_{10}$  number of parasites inoculated. The group median times are plotted in Fig. 8. The relationships are strikingly regular. For this reason the curves have been drawn by eye. The two curves are parallel but differ in constants by two days. This corresponds to the median duration of haemoglobinuria.

The gradient of the curves measured graphically is -1.0; thus a  $\log_{10}$  increase in inoculum decreases the

Table 9

The mortality rates in mice inoculated with different numbers of B. rodhaini in rat blood

No. of <u>B. rodhaini</u> inoculated	Died	Survived
$5.7 \times 10^6$	7	1
$5.7 \times 10^5$	7	1
$5.7 \times 10^4$	8	0
$5.7 \times 10^3$	8	0
$5.7 \times 10^2$	8	0
$5.7 \times 10^1$	7	1
$5.7 \times 10^0$	4	4

Fig. 5

The titration of B. rodhaini infected  
rat blood in mice

LOG<sub>10</sub> PARASITES INOCULATED

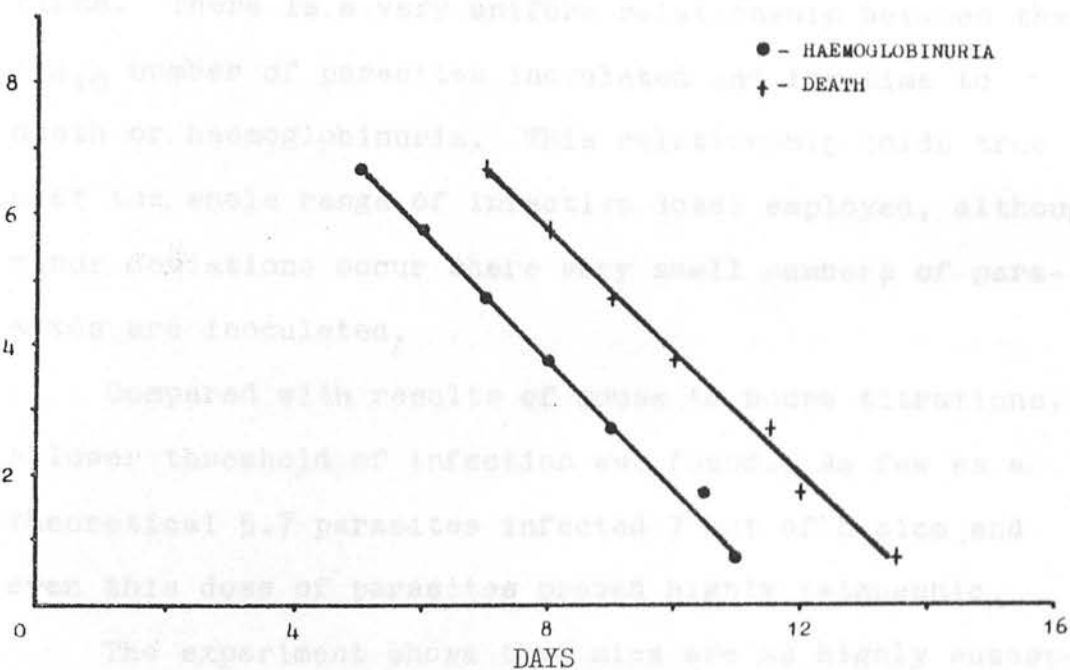


Fig. 8

The titration of B. rodhaini infected  
rat blood in mice



time to death (or haemoglobinuria) by one day.

Applying the formula given by Overdulve & Antonisse (1970), the generation time of B. rodhaini in this experiment was 7.2 hours.

### Conclusions

The results of this titration are very similar to those previously described for mouse to mouse titrations. There is a very uniform relationship between the  $\log_{10}$  number of parasites inoculated and the time to death or haemoglobinuria. This relationship holds true over the whole range of infective doses employed, although minor deviations occur where very small numbers of parasites are inoculated.

Compared with results of mouse to mouse titrations, a lower threshold of infection was found. As few as a theoretical 5.7 parasites infected 7 out of 8 mice and even this dose of parasites proved highly pathogenic.

The experiment shows that mice are as highly susceptible to primary infection with B. rodhaini in rat erythrocytes as to B. rodhaini in mouse erythrocytes. (The converse does not apply. Rats are less susceptible to B. rodhaini in mouse erythrocytes than to B. rodhaini in rat erythrocytes, McHardy, 1967). It demonstrates that the subinoculation of blood into mice is an extremely sensitive means of detecting the presence of B. rodhaini in rats. If as few as 5 parasites in 0.25 ml. of blood can be detected, and assuming a total blood volume of

10 mls. in a 200 gms. rat (from Schermer, 1967), then it follows that the presence of as few as 200 freely circulating parasites in a rat can be detected by this technique.

(v) A further series of subinoculations from *B. rodhaini* recovered rats into mice

This experiment was carried out to confirm the absence of persistent infection in rats, using the preferred detection technique of subinoculation.

Materials and methods

The rats which were used had recovered at different times from infection with *B. rodhaini* during maintenance of the parasite by passage. These rats had been involved in passages numbered 45-74. The maximum recorded primary parasitaemia in these rats was in excess of 5%, except in three, where it was 0.1%, 0.4% and 2%.

Mice were examined for twenty days after subinoculation. In this experiment they were challenged with *B. rodhaini* infected mouse blood.

Results

The results of the subinoculations are shown in Table 10. Six of twenty-four rats harboured latent infection, up to 43 days after inoculation.

Conclusions

Several rats in this series were found to retain latent infection for a time considerably longer than that which had previously been observed. Prior to this

Table 10

The persistence of B. rodhaini in rats as determined by  
the subinoculation of blood into mice

Days since inoculation	Number of rats tested	Number of rats infected
0 - 20	3	3
21 - 40	4	2
41 - 60	4	1
61 - 80	4	0
81 - 100	1	0
101 - 150	3	0
151 - 210	5	0
Totals	24	6

experiment, no recovered rat had been shown, by splenectomy or subinoculation, to be latently infected ten or more days after primary parasitaemia became sub-patent.

Rats used in previous experiments had been infected with B. rodhaini which had been passaged relatively few times compared with the B. rodhaini used to infect rats in the present experiment. It was therefore decided to investigate what effect the repeated passage of B. rodhaini might have on the persistence of the parasite in rats after recovery.

- (vi) The effect of passage on the persistence of B. rodhaini in the rat

#### Materials and methods

Thirty rats were inoculated, each with either  $6.0 \times 10^7$  or  $8.0 \times 10^7$  erythrocytes infected with a high-passage isolate of B. rodhaini (passaged 88 times in rats). Fifteen rats were inoculated, each with  $8.0 \times 10^7$  erythrocytes infected with a low-passage isolate of B. rodhaini. (This B. rodhaini had been passaged nine times in rats, frozen as stabulate TREU 1127, then passaged a further three times in rats). In order to inoculate equal numbers of parasites into the two groups, the high-passage isolate was diluted in buffered salt solution pH 8.0 (see Appendix 1).

Thin blood smears were examined from day 6 to the onset of latency, which was taken as the first day on which the parasitaemia fell below 0.1%. Blood was

subinoculated from rats into mice ten days after the onset of latency.

### Results

All rats became infected. There was no difference in reaction between rats infected with  $6.0 \times 10^7$  high-passage B. rodhaini and those infected with  $8.0 \times 10^7$ , so the results have been combined.

Parameters of the infections produced by the low-passage and high-passage isolates of B. rodhaini are summarised in Table 11. The virulence of the parasite had been increased by passage, as is indicated by a significantly higher mortality rate in the high-passage infected rats. Compared with the reaction in low-passage infected rats, that in high-passage rats was characterised by a slightly longer period of parasitaemia, in which maximum parasitaemia and the onset of latency were reached significantly later.

In spite of these differences, the groups did not differ in terms of the persistence of the parasite. No rat in either group was shown to be infected ten days after the onset of latency.

### Conclusions

It is clear from the results of this experiment that whilst passage of B. rodhaini in rats at weekly intervals will enhance the virulence of the organism, it does not increase the persistence of the parasite in those rats which recover. The difference in results from the

Table 11

Parameters of infection in rats inoculated with equal numbers of two isolates of B. rodhaini passaged for a different number of times

	Low passage	High passage	Statistical comparison
Mortality	0/15	17/30	p = <0.001 Sig.
Day of max. parasitaemia	6.1 $\pm$ 0.3	7.0 $\pm$ 1.2	p = <0.01 Sig.
Day of onset of latency	8.3 $\pm$ 0.6	10.4 $\pm$ 1.7	p = <0.001 Sig.
No. latently infected	0/15	0/13	No difference

two series of subinoculations cannot therefore be explained as being due to the infection of rats with B. rodhaini subjected to different numbers of passages.

- (vii) The effect of host age on the persistence of B. rodhaini in the rat

#### Materials and methods

Eight male and eight female rats of between 45 and 48 days of age were inoculated with  $1.4 \times 10^6$  parasitised red blood cells. Thin blood smears were examined from days 6-13.

Primary parasitaemias were regarded as sub-patent when they fell below 0.1%. Subinoculations were performed ten days after the onset of latency. Mice were examined daily for eighteen days after subinoculation.

#### Results

All the rats became infected. All eight male rats died. The median time to death was 10 days and maximum parasitaemia for the group was  $58\% \pm 9.4\%$ . Four of the eight female rats died (median time to death 10 days) and the maximum parasitaemia was  $53\% \pm 11.1\%$ . Although the mortality rate was higher in the male rats than in the female, the difference, by chi-square analysis, was not significant ( $p = > 0.05$ ). There was no difference in mean maximum parasitaemias between the male and female rats ( $p = > 0.05$ ).

Subinoculation tests from the four surviving female rats were negative.



## Conclusions

The small number of young rats which survived infection in this experiment did not harbour latent B. rodhaini ten days after primary parasitaemias became sub-patent. In this respect, the infection in young rats did not differ from that in adult rats.

### (viii) A final experiment

This experiment was set up to investigate aspects of the immunity in rats following recovery from B. rodhaini infection. The parasitaemic status of the rats after recovery was determined by subinoculation.

The author's departure to Africa prevented the experiment from being completed, but the results of the subinoculations are interesting and provocative.

## Materials and methods

Thirty-two rats were inoculated, each with  $2.8 \times 10^6$  B. rodhaini infected erythrocytes, from an isolate which had been passaged seventeen times in rats and frozen twice during this history. It was thus a low-passage isolate.

Thin blood smears were examined on days 5, 7, 9 and daily thereafter until latency. As before, the first day of latency was taken as the day on which the parasitaemia was less than 0.1%.

Blood was subinoculated from rats into mice ten days after the onset of latency. Mice were examined daily for 19 days after subinoculation and were later challenged

with B. rodhaini infected rat blood.

### Results

All the rats became infected and survived.

The results of subinoculations from five rats were not conclusive. Recipient mice showed no evidence of haemoglobinuria or death after either subinoculation or challenge. In twenty-seven rats conclusive results were obtained from the subinoculations. Nine were shown to harbour latent infection, whilst in eighteen, according to the test, a sterile state existed.

For the purpose of analysis, the rats have been divided into two groups on the basis of this result, and some parameters of the primary parasitaemias in these two groups are compared in Table 12. It is apparent that the persistence or non-persistence of parasitaemia after recovery was not related to differences in the primary parasitaemias.

### Conclusions

In terms of the persistence of infection, the results of this experiment diverge considerably from those previously described. In Table 13, a comparison is drawn between the results of the present experiment and those results of an earlier experiment, tabulated in Table 11, relating to the infection of rats with a similar low-passage isolate of B. rodhaini. It can be seen that the experiments differed in the proportions of rats shown to harbour latent infection, in the number of parasites

Table 12

Some parameters of the primary parasitaemias in rats  
shown subsequently to be either latently infected or  
sterile of infection

	Parasitaemic state after recovery		Statistical analysis
	Latent	Sterile	
Number of rats	9	18	
Max. observed parasitaemia % age	$4.3 \pm 3.6$	$8.8 \pm 9.3$	$p = > 0.1$ Not sig.
Day of max. parasitaemia	$7.4 \pm 0.9$	$8.0 \pm 1.1$	$p = > 0.1$ Not sig.
Day of onset of latency	$10.1 \pm 1.7$	$10.9 \pm 1.4$	$p = > 0.2$ Not sig.

Table 13

A comparison of the results of two experiments in  
which different proportions of recovered rats  
retained latent infection

No. of <i>B. rodhaini</i> parasitised erythrocytes inoculated	$8.0 \times 10^7$	$2.9 \times 10^6$	
Latently infected	0/15	9/27	$p = < 0.05$ Significant
Day of maximum parasitaemia	$6.1 \pm 0.3$	$7.8 \pm 1.1$	$p = < 0.01$ Significant
Day of onset of latency	$8.3 \pm 0.6$	$10.6 \pm 1.5$	$p = < 0.01$ Significant

inoculated and in certain parameters of the primary parasitaemias. These latter were probably a result of the different inoculum doses. Since differences in these parameters may not necessarily relate to the duration of infection (see Table 11), it is possible that the number of parasites inoculated influences the fate of parasites, but does so by its effect on other parameters of parasitaemia e.g. the magnitude of maximum parasitaemia.

### Discussion

The strain of B. rodhaini used in these experiments originated in mice and was adapted to rats. After adaptation, its characteristics with regard to parasitaemia and pathogenicity resembled those previously described by many workers. However, in terms of its persistence in the rat host, this strain differed markedly in that a sterile cure was the natural and usual sequel to primary infection. Phillips (1969a) and Young (1970) both found that chronic infection ceased to be detectable between 1-3 months after infection, but that a proportion of rats remained infected for up to one year. In the present study, very few rats retained detectable infection beyond ten days after the onset of latency. A few rats, however, did harbour latent infections. It was shown that the passage state of the organism did not affect the tendency for primary infection to be followed by sterile cure, even though the time course of primary parasitaemia was significantly altered. The analysis of parasitaemias from a group

of rats, some of which effected sterile cures and some of which did not, failed to relate parameters of the primary parasitaemia to its fate. Young rats which survived infection were also able to effect a sterile cure.

It is apparent from this and other quoted studies that the fate of B. rodhaini in rats is not consistent. From examination of the experiments compared in Table 13, it is apparent that the number of parasites inoculated differed by about  $1\frac{1}{2} \log_{10}$  increments. It is possible that a smaller inoculum dosage of parasites provokes a less severe host response which allows parasites to persist. A notable feature of the infections was the marked morphological change in parasites which occurred at crisis (Plate 5a) in which the appearance of parasites closely resembled that seen after drug treatment (Plate 5b). This feature has not been remarked upon by other workers studying rat infections in which persistent infection was the normal sequel to recovery, and its presence could be associated with the sterile cures which were observed. Studying Plasmodium knowlesi infections in monkeys, Brown, Brown & Hills (1970) noted that some immunised animals effected a sterile cure of the challenge infection, and in these cases abnormal forms of the parasite were characteristically present at the crisis of the challenge parasitaemias.

In contrast to the findings of Phillips (1969a), the technique of subinoculation was found to be more sensitive than splenectomy in detecting latent infections. It has

the advantage that the test animal is relatively unaffected by the procedure. Moreover, by making use of the fact that about 95% of all mice infected with B. rodhaini will exhibit haemoglobinuria, a great deal of microscopic examination is eliminated. In addition, the reaction time in subinoculated mice will give an estimate of parasitaemia in the donor blood. All these considerations make subinoculation an attractive technique.

Subinoculation has been shown to be an extremely sensitive detection method. An interesting finding was that, in mice, the minimum infective dose of B. rodhaini infected rat blood was less than that of B. rodhaini infected mouse blood. The difference might be due to a difference in the parasite or to a difference in diluents for the two titrations. The titration of infected rat blood utilised non-infected blood as the diluent medium. This may have been less harmful to the parasites than the buffered salt solution used in mouse to mouse titrations.

Some difficulty was occasionally encountered in deciding the results of subinoculation tests when challenged mice did not show 'redwater'. This difficulty was found to be associated with challenge by infected rat erythrocytes, and appears to be a manifestation of the effect described by Ludford (1967, cited in Mahoney, 1972) who showed that mice were protected from infection with infected rat erythrocytes by antiserum against normal rat erythrocytes. The problem was much reduced by challenging

subinoculated mice with infected homologous erythrocytes.

Enhancement of the virulence of Babesia spp. by passage is a well established feature of the parasite. Working independently with the same strain of B. rodhaini, Dolan (1974) also noted the phenomenon. The increase in virulence of B. rodhaini in mice, since its original isolation, has already been discussed and Cox & Turner (1970) remark that the relatively non-pathogenic B. microti can become, after rapid passage, lethal for mice. The lack of stability of piroplasms which are constantly syringe passaged has prompted a number of workers to stress the need for preserving piroplasms at low temperatures as stabilates (Lumsden & Hardy, 1965).

#### c) Immunity in recovered rats

##### Introduction

Experiments which have been described have demonstrated that nearly all rats infected with B. rodhaini were no longer infected, as determined by subinoculation or splenectomy, within ten days of the primary parasitaemia becoming sub-patent. Some of these rats have been the subject of an experiment to determine if, following this natural sterile cure, they were immune to subsequent re-infection.

##### Materials and methods

Twenty-four rats which had all been shown, by subinoculation, not to be infected ten days after the primary parasitaemia became sub-patent, were challenged in



two groups, one group at 120 and the other at 370 days after infection, with  $10^8$  B. rodhaini infected rat erythrocytes. Five rats, of the same age as the test rats, but which had never been infected with B. rodhaini, were infected, at each challenge, as controls.

Blood smears made daily from each rat were examined for 18 days after challenge, and  $10^4$  erythrocytes in each smear were scanned.

### Results

The two groups of five control rats all became infected and showed patent parasitaemias after challenge, (mean maximum parasitaemias  $19.4\% \pm 17\%$  and  $48.5\% \pm 8.4\%$ ) which were typical of primary parasitaemias. All of the rats recovered.

Following challenge at 120 days after primary infection, one rat of a group of 13 showed a low transient parasitaemia (maximum 0.5%), whilst in the other twelve rats, a patent parasitaemia was not observed.

At 370 days after primary infection, none of a group of 11 rats developed a patent parasitaemia after challenge.

### Conclusion

The experiment shows that rats remain immune to challenge after a natural sterile cure of infection, and that this sterile immunity will be maintained for at least a year.

### Discussion

Classically the protective immunity in babesiosis

has been associated with the continuing presence of parasites. However, in recent years, there has been a growing body of evidence that a true sterile immunity to many species including B. divergens (Davies, Joyner & Kendall, 1958; Joyner & Davies, 1967) and B. bigemina (Callow, 1964 and 1967), can exist.

The present findings add to this evidence, and confirm the results of Phillips (1968), who observed sterile immunity in rats which had recovered from B. rodhaini both naturally and after drug treatment. The protection lasted for up to 12 months, which is in agreement with our own findings.

Very few other references to a natural state of sterile immunity following B. rodhaini infection are recorded. Colas-Belcour & Vervent (1953) have recorded that, in some mice which survived B. rodhaini infection, a sterile immunity occurred.

In the present experiment, rats were challenged with the same stabilate which had been used to infect them. Callow, McGregor, Parker & Dalgliesh (1974) have shown that sterile cured cattle are immune to challenge with heterologous as well as with homologous strains of B. argentina, but that the degree of immunity appears to be related to the range of antigenic variation to which the hosts are exposed.

In view of the current interest in sterile immunity in Babesia spp. infections, the infection of rats with

B. rodhaini provides an excellent model system for studies of this phenomenon since a sterile cure occurs without the use of drugs. A study of the circumstances which lead to a natural sterile cure rather than to a persistent latent parasitaemia could yield some very interesting and useful results which could be pertinent to several practical situations e.g. the use of living vaccines.

The parasite is a highly virulent, fast growing, and easily cultured organism. It is a member of the family of *Trypanosoma*, the present author has been studying it for several years. It is a blood protozoan and is found in the blood of its host. It is a flagellate and has a long flagellum. It is a unicellular organism and is found in the blood of its host. It is a blood protozoan and is found in the blood of its host. It is a flagellate and has a long flagellum. It is a unicellular organism and is found in the blood of its host.

Shortt & Blake (1961) have reported isolated from British rodents and parasites from the laboratory host for study. Since then a number of workers have reported on the morphology and characteristics of infection of B. rodhaini in both mice and rats (Cox & Young, 1969; Russell, 1969; Young, 1970; Clark & Young, 1970).

This work demonstrates that the majority of both mice and rats recover from infection and become latent parasitaemic (although Cox & Young, 1969, showed that rapid passage can take the parasite from mice).

In his extensive studies of the parasite, Young

C. Babesia microti in mice

Introduction

B. microti was first described from Microtus incertus in Portugal by França (1910). Similar parasites have since been recorded in a variety of small mammals in Europe and America. Cox (1970) has listed the mammalian hosts for B. microti in Britain and Young (1970) found the parasite to be widely distributed wherever he collected rodents in the South of England and in Ireland. The present author took the opportunity of examining blood from rodents captured around Edinburgh and kindly made available by Mrs. M. Bathgate, Zoology Department, University of Edinburgh. B. microti was observed in blood of 2/12 short-tailed voles (Microtus agrestis), but not in smears from 39 bank voles (Clethrionomys glareolus) or 9 long-tailed field mice (Apodemus sylvaticus).

Shortt & Blackie (1965) made several isolates from British rodents and passaged them into laboratory hosts for study. Since then a number of workers have reported on the morphology and characteristics of infection of B. microti in both mice and rats (Cox & Young, 1969; Nowell, 1969; Young, 1970; Clark & Allison, 1974).

This work demonstrates that the majority of both mice and rats recover from infection and harbour latent parasitaemias (although Cox & Turner, 1970, comment that rapid passage can make the parasite lethal for mice).

In his extensive studies of the parasite, Young

(1970) succeeded in transmitting B. microti transtadially with Ixodes trianguliceps.

In spite of the fact that B. microti was discovered over fifty years ago, it has received relatively little attention in comparison with B. rodhaini. This imbalance will possibly be corrected since more recent studies e.g. Lykins, Ristic, Weisiger & Huxsoll (1975), have been stimulated by the involvement of B. microti in human infections (Gleason, Healy, Western, Benson & Schultz, 1970).

#### a) Characteristics of infections in mice

##### Materials and methods

These results have been collated from observations made during the maintenance by passage of the parasite, and from mice infected in groups for specific experiments.

##### Results

Mice were consistently and easily infected. Parasitaemias were high and prolonged, and fell slowly to sub-patent levels. The mortality rate was very low, although at the height of infection a proportion of mice was observed to be ill. Some of these showed a green discoloration of the urine but frank haemoglobinuria was rare.

Data from a group of fifteen mice simultaneously infected with  $1.9 \times 10^7$  infected erythrocytes are typical. The maximum parasitaemia ( $55.3\% \pm 18\%$ ) was reached nine days after infection. This slowly fell to below 0.1% in

most mice, between 32 and 45 days post-infection.

In nearly all mice which were observed for a sufficient length of time, the primary parasitaemia did not decline evenly, but instead, subsidiary peaks of parasitaemia of diminishing magnitude occurred (Fig. 9). The relapse interval was  $7.6 \pm 1.9$  days (mean and standard deviation of 21 observations).

Multiple parasitism of erythrocytes was common at higher parasitaemias and the so-called 'Maltese Cross' forms were occasionally seen. The morphology of the parasite was as previously described. Spectacular changes in morphology during recovery were not observed.

b) The persistence of infection in mice and immunity to re-infection

Materials and methods

Blood smears were prepared from 36 mice which had recovered at different times from inoculation with  $10^7 - 10^8$  parasitised erythrocytes during the maintenance of B. microti by passage.

Eighteen of these mice, together with five previously non-infected mice as controls, were then challenged with  $2.6 \times 10^7$  B. microti infected erythrocytes. Blood smears from the mice were examined daily for 20 days after challenge.

Of the remaining 18 mice, seven mice which did not have patent parasitaemias when examined, were killed and blood from each was subinoculated into pairs of mice.

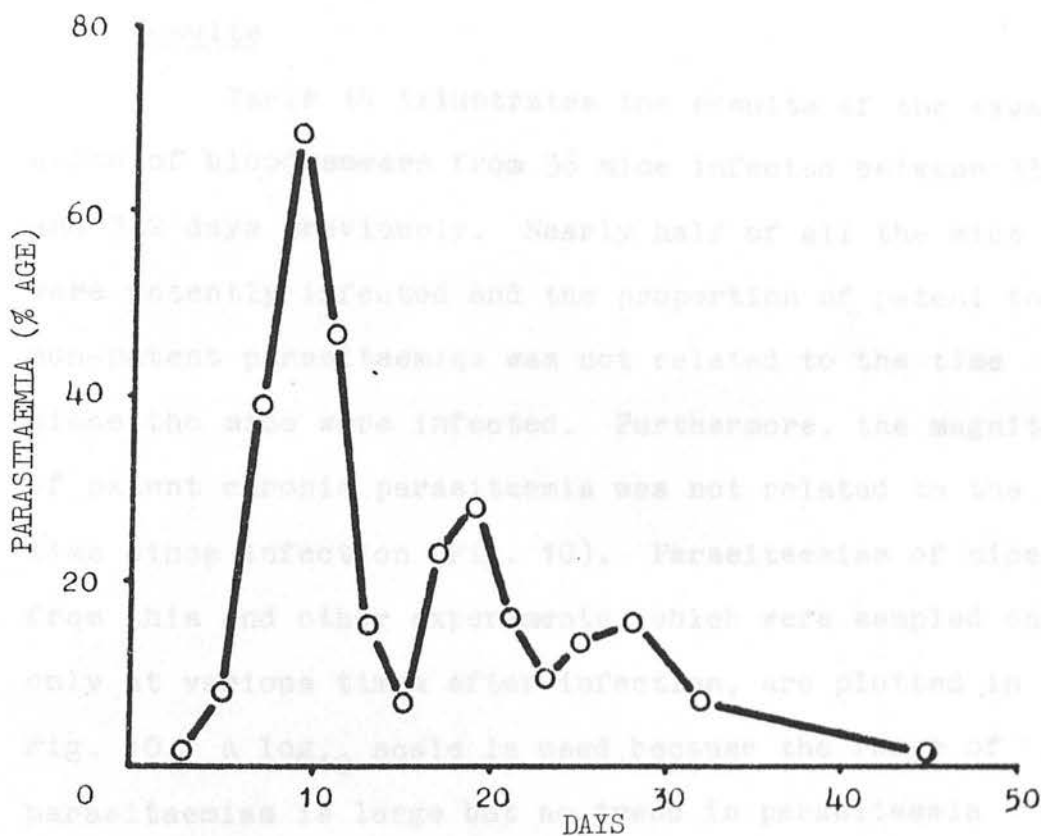


Fig. 9

A typical parasitaemia curve of B. microti  
in a mouse



Blood smears from the subinoculated mice were examined daily from days 9-21 following subinoculation.

In all the blood smears examined,  $10^4$  erythrocytes were scanned. Thus the level of patency in this experiment was 0.01%.

### Results

Table 14 illustrates the results of the examination of blood smears from 36 mice infected between 33 and 322 days previously. Nearly half of all the mice were patently infected and the proportion of patent to non-patent parasitaemias was not related to the time since the mice were infected. Furthermore, the magnitude of patent chronic parasitaemia was not related to the time since infection (Fig. 10). Parasitaemias of mice, from this and other experiments, which were sampled once only at various times after infection, are plotted in Fig. 10. A  $\log_{10}$  scale is used because the range of parasitaemias is large but no trend in parasitaemia against time is evident.

Of the seven mice from which blood was subinoculated, two were found to be latently infected. The five mice not shown to be latently infected had been infected between 130 and 316 days previously.

Of the challenged mice, seven had patent parasitaemias at the time of challenge. In one of these, the parasitaemia rose slightly after challenge, and, in the other six, patent parasitaemias were not affected by challenge.

Table 14

The persistence of patent parasitaemias in B. microti  
infected mice

Days since infection	Number with patent parasitaemia	Number without patent parasitaemia
33 - 50	1	2
51 - 100	5	2
101 - 150	1	4
151 - 200	1	3
201 - 250	3	2
251 - 300	2	2
300 - 322	4	4
Total	17	19

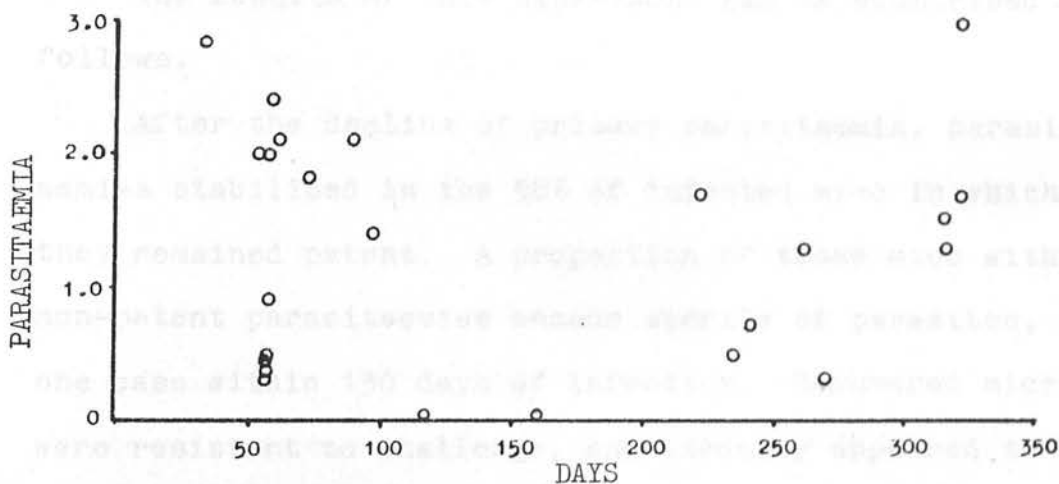


Fig. 10

Scatter diagram illustrating levels of

patent parasitaemia in mice at various

times after *B. microti* infection

(Parasitaemia =  $\log_{10}$  parasitised r.b.c.s/10<sup>4</sup> r.b.c.s)

Eleven mice, for which blood smear examination had been negative, were challenged, and in seven of these a patent parasitaemia of low magnitude appeared at varying times after challenge. The parasitaemias in these mice were of much lower magnitude than those in control mice, ( $1.7\% \pm 1.9\%$  and  $46.2\% \pm 21.6\%$  mean maximum parasitaemia and standard deviation of the two groups respectively;  $p = < 0.01$ , significant).

The results of this experiment can be summarised as follows.

a) After the decline of primary parasitaemia, parasitaemias stabilised in the 50% of infected mice in which they remained patent. A proportion of those mice with non-patent parasitaemias became sterile of parasites, in one case within 130 days of infection. Recovered mice were resistant to challenge, and immunity appeared to be related to the level of chronic parasitaemia. If this was patent, immunity was complete, but if it was non-patent, a proportion of mice (perhaps those which were no longer latently infected?) were only partially protected.

c) The effect of B-methasone in B. microti recovered mice

#### Materials and methods

Fifty-eight days after infection with B. microti fifteen mice were randomly divided into two groups. Eight mice were given five daily intramuscular injections of B-methasone ('Betsolan', Glaxo, Greenford, Middlesex at

5 mgms./kgm. weight) with 0.05 ml. of normal saline, and seven mice received a corresponding series of injections of normal saline alone.

Thin blood smears were examined daily from day 0 (the first day of treatment) for 24 days, and  $10^4$  erythrocytes were scanned in each smear.

### Results

The range of parasitaemias on day 0 was large. Three mice did not have patent parasitaemias, whilst the rest had patent parasitaemias at low levels (range 0.01% - 0.08%) except for two mice with parasitaemias of 1% and 2.6%.

Treated mice tolerated the dose of B-methasone well without ill-effects. The effect of B-methasone treatment on the chronic parasitaemias was difficult to evaluate, due partly to the widely varying initial parasitaemias, and also due to the fact that several control mice showed an increased parasitaemia during the experiment. Various parameters of parasitaemias in the two groups were compared by non-parametric statistical analysis. In both the treated and the untreated groups, the initial parasitaemias were exceeded later in the experiment. Subjecting these data to Wilcoxon's Matched Pairs Signed Rank test the difference was significant for both groups ( $p = < 0.05$ ).

Between the treated and the untreated groups, no difference at the 5% level of probability existed in;

initial parasitaemia, maximum post-treatment parasitaemia, difference between initial and maximum parasitaemia, index of response (maximum parasitaemia/initial parasitaemia), and cumulative increase in parasitaemia - by White's test, and  $\log_{10}$  index of response - by 't' test.

Of the three mice without patent parasitaemias at the beginning of the experiment, two received B-methasone, and, in one of these, parasites were later observed. The third mouse received control injections, and later showed a low and transient parasitaemia.

### Discussion

The 'droplet' type of morphology seen in B. rodhaini in rats at crisis was not seen in mice infected with B. microti, and indeed no notable morphological changes were observed. Clark, Wills, Richmond & Allison (1975) have remarked upon the appearance of intra-erythrocytic inclusion bodies in mice at the crisis of B. microti parasitaemia. They consider these bodies to be pyknotic remnants of killed parasites. Young (1970) probably observed these when he noted the appearance of small forms of parasite at crisis, and in our own studies of bovine Babesia spp., similar changes have been noted (see later). Clark, Wills, Richmond & Allison (1975) argue that their evidence of intra-erythrocytic parasite death suggests that a non-antibody, soluble anti-parasitic agent is produced by host animals.

Recrudescent parasitaemias during the declining phase

of parasitaemia were a feature of infections studied in the present work. Similarly, using the same strain of B. microti, Gamble (1974) observed a secondary and lesser parasitaemic peak eight days after the peak of primary parasitaemia. In general, relapsing parasitaemias have not been described by others working with B. microti in mice (Cox & Young, 1969; Clark & Allison, 1974), in rats (Nowell, 1969), in hamsters (Wolf, 1974; Lykins, Ristic, Weisiger & Huxsoll, 1975) and voles (Van Peenen & Healy, 1970), although Young (1970) infers that recrudescent parasitaemias were occasionally observed in his recovering mice.

In other respects the characteristics of parasitaemia resembled those reported in the literature. In spite of high maximum parasitaemias, B. microti caused very few deaths and only a mild degree of illness. This most interesting feature of the organism was examined by Gamble (1974) who compared the haematological changes in mice produced by B. microti and B. rodhaini. He observed that not only were parasitaemias of B. microti as high as those of B. rodhaini, but also that a similar degree of anaemia was produced by the two organisms. This implies that the lethal effect of B. rodhaini is due to factors other than its ability to cause anaemia.

Young (1970) found that infections persisted for up to a year at sub-patent levels in a decreasing proportion of mice. The results of our own experiments are more



akin to those of Irvin & Brocklesby (1969) who described infections of both splenectomised and non-splenectomised mice. They encountered chronic parasitaemias which were at higher levels in splenectomised than in non-splenectomised mice, and which persisted for at least a year after infection. Similar parasitaemias were observed after the infection of nude hypothyroid mice (Clark & Allison, 1974) and of hamsters given antilymphocytic serum (Wolf, 1974). In our experiments, neither the frequency of patent parasitaemia, nor its magnitude, showed any trend in relation to time beyond approximately 50 days after infection. This suggests that the host-parasite relationship of mice and B. microti reaches a stable equilibrium after primary parasitaemia. The level at which the parasitaemia stabilises appears to be determined by the immunological competence of the host. Should this be impaired by splenectomy, congenital thymic deficiency or the administration of antilymphocytic serum, then equilibrium is maintained at a higher level than would otherwise be the case.

The level of protective immunity was found to be related to the level of parasitaemia. Mice without patent infection were still protected to a varying degree against challenge, but less so than patently infected mice. By comparison, Cox & Young (1969), on rather limited evidence, regard the presence or absence of infection as of no influence on the immunity of rodents to B. rodhaini and B. microti.

The investigation of the effects of B-methasone on B. microti highlights the difficulties in evaluating attempts to provoke relapse parasitaemias in a host-parasite situation which is a dynamic equilibrium characterised by a fluctuating parasitaemia. With any fluctuating parasitaemia, it is likely that the level of parasitaemia at some stage of the period of observation will exceed that on the first day of observation and this was found to be the case. However, periodic relapses of parasitaemia were not observed, and injected corticosteroid had no significant effect. This is in accordance with the results of Young (1970), when investigating chronic infections, although recrudescent parasitaemias were produced by B-methasone given when the primary parasitaemia was waning (Young & Cox, 1971). These workers successfully provoked recrudescent B. microti parasitaemias in a proportion of chronically infected mice and voles, which led them to suggest that the elevated corticosteroid levels known to occur in wild voles under stress may provoke relapse parasitaemias of piroplasms and perhaps disease. The cogency of this suggestion is reasonable, but, in view of the inconsistent ability of B-methasone to provoke relapse parasitaemias, more experimentation is required. Additional evidence that immunosuppression can produce B. microti relapse parasitaemias was provided by Wolf (1974), who observed recrudescent parasitaemias following the administration of anti-

lymphocytic serum to latently infected hamsters.

B. microti infection in mice resembles the Babesia spp. infections of veterinary interest in a number of ways, in that the mouse is a natural host, most mice survive infection, persistent parasitaemia succeeds acute infection, and the organism is transmissible by ticks. In view of the close analogy, this host-parasite system merits far more study than has been accorded to it in the past. A good deal of current research on B. microti has been stimulated by its involvement in human cases of babesiosis. This should not obscure the fact that the organism offers an excellent model system for the study of babesiosis of veterinary significance, especially in those areas, such as antigenic variation, which are related to chronic parasitaemias.

## EXPERIMENTS ON CATTLE

### Introduction

The intention was to study chronic parasitaemias of B. divergens in Britain and of B. bigemina in Nigeria and to determine the effects of B-methasone and A.C.T.H. on these parasitaemias. In this way, it was hoped to confirm or reject the hypothesis that stress provokes relapses of babesiosis, at least in part, through the action of raised endogenous corticosteroid levels.

In some experiments plasma cortisol levels were measured directly. This was not always possible and so eosinophil, lymphocyte and neutrophil counts were regularly performed. These leucocyte parameters are good indirect indicators of the presence of corticosteroids in the blood (Hopwood & Tibolla, 1958; Pehrson & Wallin, 1966 and others) and are altered in cattle under stress (Kerr, Robertson & McGirr, 1951; Merrill & Smith, 1954; Volker, Furcht, Stolpe & Bauer, 1973). They were used to provide evidence that the injected B-methasone or A.C.T.H. was potent, to give a measure of the physiological effect of these drugs and as a means of monitoring spontaneous changes in plasma cortisol levels.

The opportunity was taken to make observations in connection with the experimental primary infections which, in the case of the I.F.A. response to B. divergens and the pathogenic effects of a Nigerian isolate of B.

bigemina, would provide original information.

The experiments in Nigeria first required that an isolate of B. bigemina be obtained, and the isolation and purification of this organism are described at the beginning of the section on B. bigemina.

We begin with an evaluation of three techniques which were of particular importance in the ensuing studies.

A. The evaluation of some important techniques

a) Acridine orange staining

Winter (1967) described the use of acridine orange to stain B. bigemina and B. argentina, and claimed that low parasitaemias of these parasites could be efficiently detected by this method. Since it was applicable to thin smears and could allow rapid quantification of low parasitaemias, the technique was thoroughly investigated. In a modified form, it was extensively applied as the standard staining method in the experiments described in later chapters.

The staining method used by Winter (1967) involved fixation in acidified methanol, passage of the fixed smear through ethyl alcohol concentrations and then alternate dipping in distilled water, acetic acid, distilled water, McIlvane's buffer, acridine orange and finally McIlvane's buffer again.

It was quickly found that this rather involved staining method was unnecessary, and the very simple and rapid method described in Materials and Methods was found to give excellent results (Trees, 1974). In the course of the development and application of this method the effect of various procedures was investigated and noted. The method was assessed for its accuracy in quantifying very low parasitaemias.



The appearance of the cellular constituents of blood and haemoparasites with acridine orange staining

In order reliably to use the stain to detect parasites in the blood, it was of great importance to know firstly how the non-parasitic constituents of blood were stained.

Acridine orange specifically stains nucleic acid.

Under the correct conditions, ribose-nucleic acid (R.N.A.) will stain orange, and de-oxy ribose nucleic acid (D.N.A.) will stain green-yellow (Udenfriend, 1969). It is this feature which makes acridine orange particularly useful for staining blood smears for haemoparasites, since normally blood contains nucleic acid only in the leucocytes. Parasites which contain nucleic acid are thus conspicuously stained amongst the erythrocytes.

The staining method described in Materials and Methods stained erythrocytes dull green against a black background. This enabled erythrocytes to be distinguished and counted. Thrombocytes appeared pale orange-green. Lymphocytes stained with a bright orange cytoplasm and a green-yellow nucleus. The nucleus of neutrophils stained green-yellow and the cytoplasm appeared a similar but much paler colour (not orange). Immature erythrocytes which contained some R.N.A., took on the orange stain. Some stained uniform dull orange and in others, orange particles were present in an otherwise dull green cell. These forms corresponded to the polychromatophil and 'stippled' cell respectively of Giemsa staining (see Plate 6). Normoblasts were small and

stained intensely with a bright yellow nucleus and a small area of orange cytoplasm. Howell-Jolly bodies were revealed as single round apple-green bodies placed eccentrically in the red cell.

Babesia spp. parasites stained with an orange cytoplasm which sometimes masked the green-yellow nuclear staining (Plates 7 and 8). Other haemoparasites stained conspicuously. Eperythrozoon spp. (Plates 9 and 10), Anaplasma marginale (Plate 11) and Borrelia theileri were a uniform bright orange. A nucleus was not present in these parasites. Theileria mutans characteristically appeared as a small orange dot and a larger pale green 'tail'.

Artifacts which might have caused confusion with parasites were rare. Nuclear fragments from 'smudged' leucocytes sometimes resembled parasites, but could be distinguished without difficulty.

#### Effect of fixation

It was found that to obtain the correct staining of erythrocytes against a black background, fixation in methanol should not exceed 2 minutes, and that after fixation the smears should be thoroughly air-dried. Failure to observe these precautions resulted in staining of the background between erythrocytes and, in extreme cases, failure of the erythrocytes to stain.

#### Effect of staining and rinsing times

These times were found to be to some extent arbitrary

Plate 6

Immature erythrocytes stained with acridine orange.

Note the normoblast and a single Babesia  
divergens parasite.

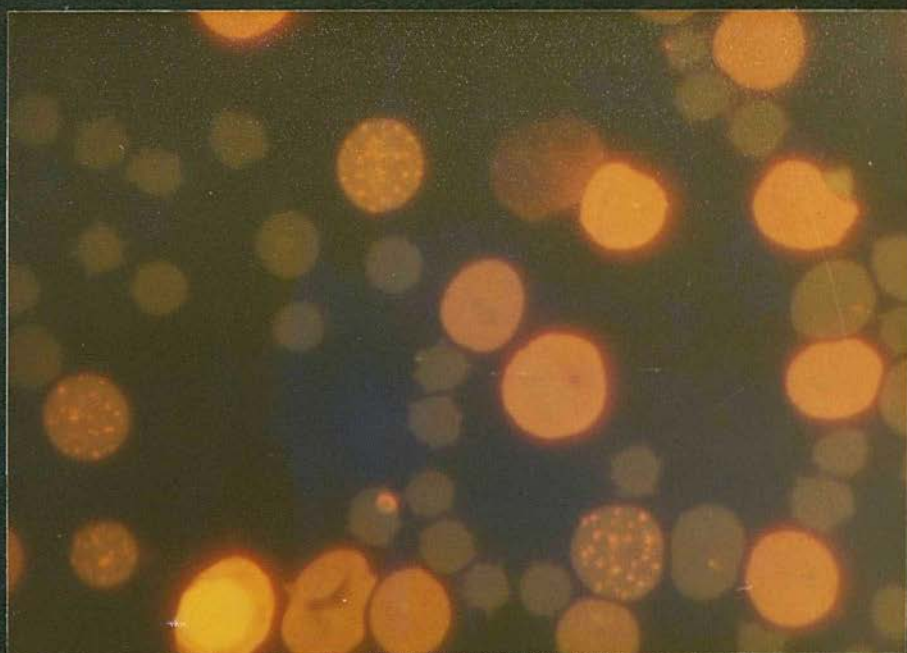


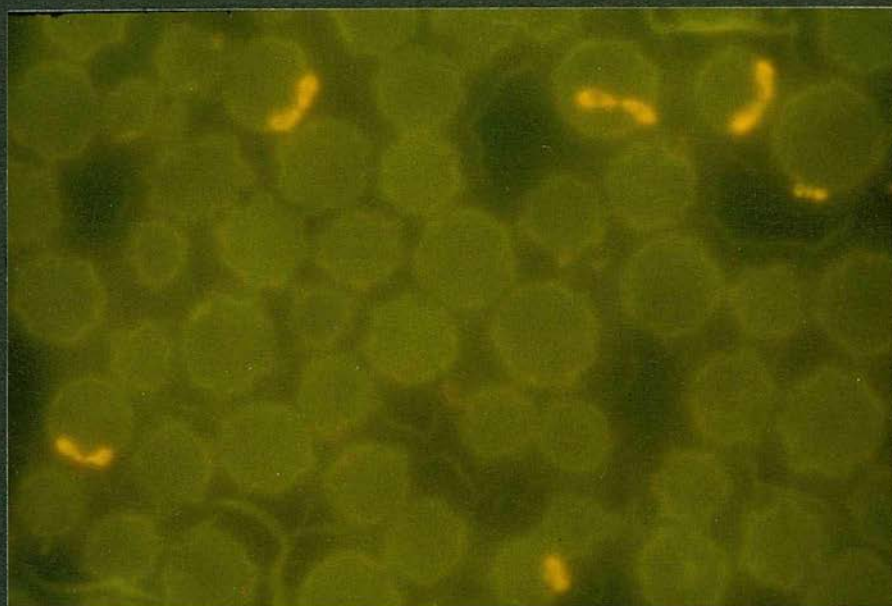


Plate 7

Babesia divergens stained acridine orange

Plate 8

Babesia bigemina stained acridine orange





Some haemoparasites stained with acridine orange

Plate 9

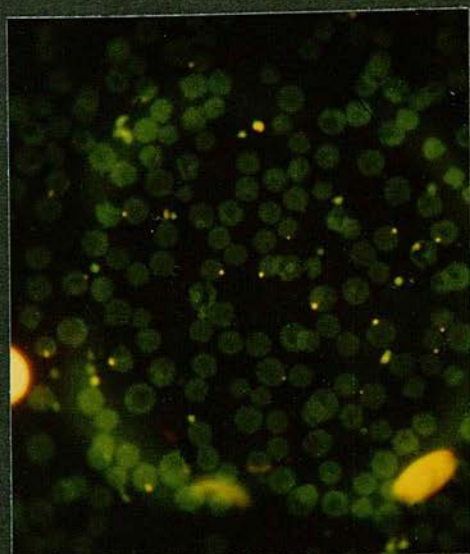
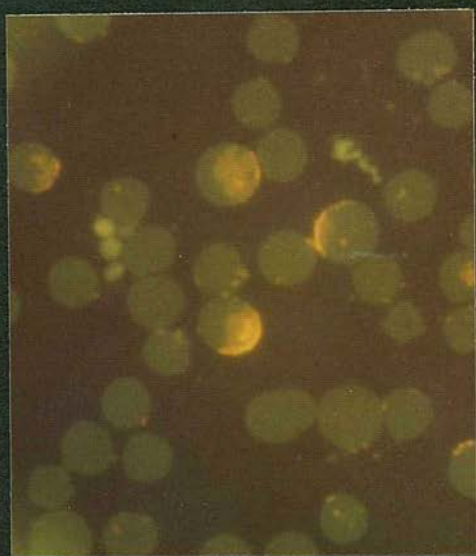
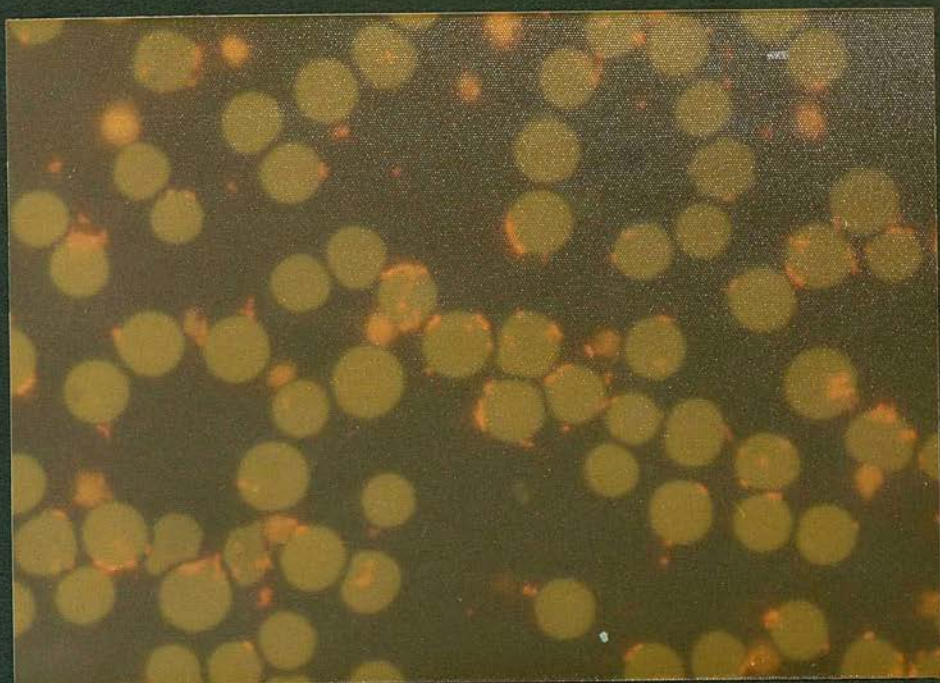
Eperythrozoon teganodes

Plate 10

E. wenyoni

Plate 11

Anaplasma marginale



and could be varied to give optimum results.

Increasing staining time increased the level of non-specific staining more than specific staining, so that a short staining time was to be preferred.

Rinsing in McIlvane's buffer differentiated nuclear and cytoplasmic staining. With brief rinsing leucocytes (and also parasites) were not well differentiated. Excessive rinsing left very weak staining and barely perceptible erythrocytes.

#### Effect of pH of buffer

Raising the pH of the buffer to pH 6.8 and pH 7.2 decreased the intensity of staining of nucleic acid and reduced the contrast between specifically stained and non-specifically stained constituents.

#### Effect of addition of sodium azide

Stock staining solution made up without the addition of sodium azide quickly became contaminated with bacteria and fungi which would in turn be transferred to smears during staining. The addition of a crystal or two (about 0.01%) of sodium azide to the stock solution prevented this and did not affect the quality of staining.

#### The storage of slides

Because of the difficulty on some occasions in staining and examining smears on the day of collection, the optimum method of storing smears was investigated. It was found that best results were obtained by storing smears unfixed and unstained. They could then be fixed and



stained in the normal manner when convenient.

### Cleanliness

As with making smears for other methods of staining, a high standard of cleanliness had a beneficial effect on the resulting quality of the stained preparation. Blood drawn aseptically into 'Vacutainers' was ideal in this respect. Smears were made on grease free and freshly dusted slides.

### Overstaining

If Giemsa stained smears were decolourised for 30 minutes in methanol they could be successfully stained with acridine orange.

### The accuracy of counts

Replicate smears were made from blood with a low parasitaemia of B. divergens. The parasitaemia in each smear was assessed by scanning  $10^5$  r.b.c.s so that a standard error for this method of counting could be derived. With a mean parasitaemia of 0.013% (13 in  $10^5$  r.b.c.s) the standard error was  $\pm 0.001$  (or 1.1 in  $10^5$  r.b.c.s). A repeat experiment with a mean parasitaemia of 0.085% gave a standard error of  $\pm 0.006\%$ .

### Efficiency

Because of the good contrast between parasites and erythrocytes, smears could be examined with medium power oil objectives. Furthermore each smear could be examined in under five minutes. This greatly reduced observer fatigue and facilitated the daily examination of blood

smears from groups of cattle for prolonged periods of up to  $3\frac{1}{2}$  months.

b) Plasma cortisol determination

In order to confirm the activity of injected A.C.T.H. and in an attempt to correlate levels of plasma corticosteroids with relapse provocation, a method of assaying plasma corticosteroids was selected.

The estimation of endogenous corticosteroids is of diagnostic importance in human conditions of the adrenal-hypophyseal system and consequently several assay techniques are available and are being constantly improved. They have been applied to certain domestic animals largely in the course of physiological research, but increasingly now the assay of plasma corticosteroids is being conducted in applied veterinary research.

The earliest methods of biological assay were superseded by the chemical method of Silber & Porter (1957), which in turn has been discarded in favour of the fluorimetric method of Mattingly (1962). This method is quicker and simpler than the Porter-Silber method and requires only 0.5 ml. of plasma.

More recently a competitive protein binding technique (Bassett & Hinks, 1969) has been developed. It is extremely sensitive but was regarded as too complex a technique to set up for the purpose of this study.

Consequently the fluorimetric method of assay was adopted. Both Mattingly (1962) and the M.R.C. Working

Party (1971) used heparin as anticoagulant in the collection of blood. Since for most of our purposes E.D.T.A. was a more suitable anticoagulant, a small experiment was conducted to determine if plasma separated from E.D.T.A. blood gave different results in the assay from blood collected in heparin.

It was intended by the administration of A.C.T.H. to elevate endogenous corticosteroid levels. In order to simulate periods of stress such as calving, transport and intercurrent disease which might persist for several days, and to provide the maximum opportunity for Babesia spp. parasitaemias to respond, the aim was to raise cortisol levels continuously for four days. A preliminary experiment on a non-infected calf was carried out to assess the degree and persistence of corticosteroid elevation produced by a single dose of A.C.T.H.

A comparison of E.D.T.A. and heparin as anticoagulants for plasma cortisol assay

Paired blood samples were obtained from a series of calves into E.D.T.A. and heparin. The cortisol concentrations in plasma from each source were compared within pairs in a sequential test. With fourteen paired estimations there was no significant difference ( $p = > 0.05$ ) in the values given by the two methods.

As a result only one blood sample into E.D.T.A. was taken in subsequent experiments and this provided all the material for haematological, parasitaemic and cortisol



estimations.

For seven non-splenectomised, 3 month old calves the mean plasma cortisol concentration was  $2.3 \pm 0.6 \mu\text{gms./100 mls.}$  No directly comparable figures have been found in the literature. Eberhart & Patt (1971) give a value of  $1.1 \mu\text{gms./100 mls.}$  in 12 day old calves by fluorimetric determination and this would be expected to increase with age.

The plasma cortisol response in a calf injected with A.C.T.H.

The response of a calf to a single injection of 40 i.u./100 kgms. wt. is shown in Fig. 11, together with simultaneous estimations from a control animal injected with normal saline.

The results of this pilot experiment show that two hours after A.C.T.H. administration plasma cortisol was raised to a maximum level four times that of the pre-treatment level. This increase in plasma cortisol was not sustained for 24 hours.

For future experiments it was decided in the first instance to administer two daily doses of A.C.T.H. in an attempt to produce a sustained elevation of plasma cortisol. Assays would be conducted on plasma collected two hours after injection of A.C.T.H.

c) The Indirect Fluorescent Antibody test

After reviewing the literature, this test was selected as a means of assessing the antibody response in

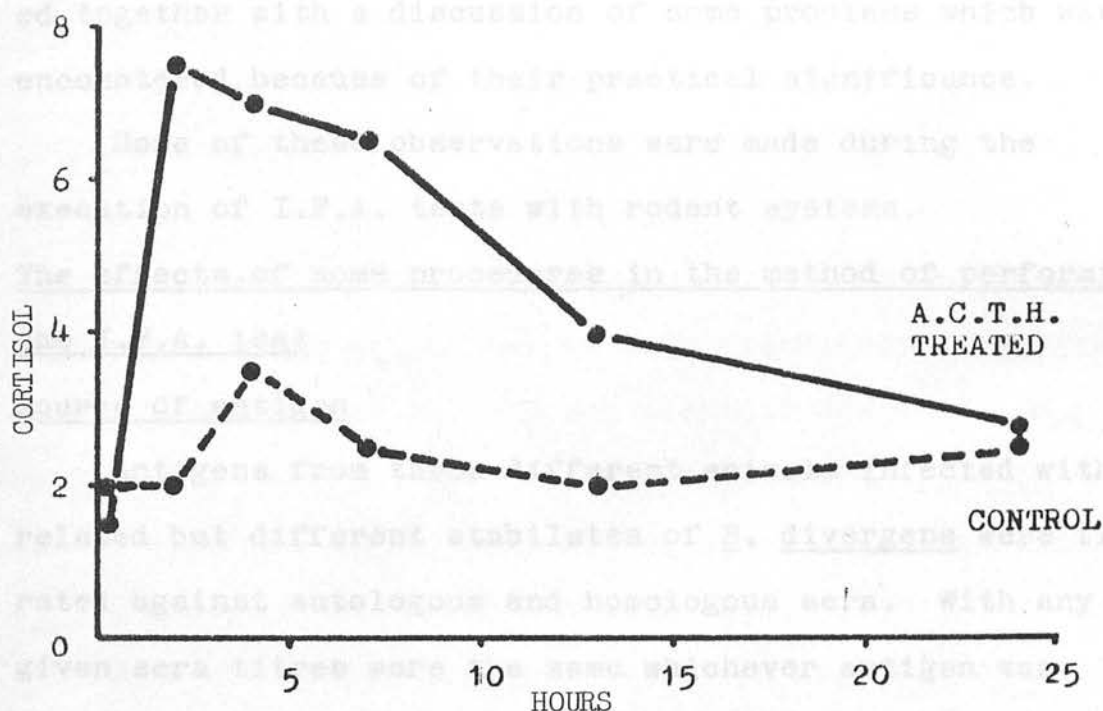


Fig. 11

Plasma cortisol concentrations (in  $\mu$ gms./100 mls.) in a calf following a single injection of A.C.T.H. (40 i.u./100 kgms. weight). Simultaneous estimations from a control calf are also shown.

cattle to primary and chronic infections and to immunosuppressant drugs and recrudescent parasitaemias.

During the course of the establishment and execution of the test the effects of certain procedures in methodology were assessed, and the reproducibility and accuracy of the test was evaluated. These observations are presented, together with a discussion of some problems which were encountered, because of their practical significance.

Some of these observations were made during the execution of I.F.A. tests with rodent systems.

The effects of some procedures in the method of performing the I.F.A. test

#### Source of antigen

Antigens from three different animals infected with related but different stabilates of B. divergens were titrated against autologous and homologous sera. With any given sera titres were the same whichever antigen was used.

#### Collection of blood into E.D.T.A. for antigen

Ross & Löhr (1968) and most other authors prepared antigen by collecting infected blood without a specific anticoagulant into large volumes of phosphate buffered saline. The ratio of buffer to blood required to prevent coagulation is about 10:1 so that this method involves handling and centrifuging fairly large volumes of fluid. By collecting blood for antigen into E.D.T.A. 'Vacutainers', the collection and washing of antigen was much

simplified.

The use of antigen collected in this way had no apparent deleterious effect on the results obtained. Titres of sera against antigen prepared in this way did not differ from titres obtained using the same sera against antigen prepared without specific anticoagulant.

#### The storage of antigen

Information on the stability of antigen at different storage temperatures is not available for Babesia spp. It is, however, of some practical significance. Ideally, the storage of antigen below about  $-40^{\circ}\text{C}$  to  $-50^{\circ}\text{C}$  is to be preferred, since biological material is likely to be stable almost indefinitely below these temperatures (B.M. Honigberg, pers. comm.). Most authors have stored Babesia spp. antigen for the I.F.A. test either in solid carbon dioxide at  $-70^{\circ}\text{C}$  or in the vapour phase of liquid nitrogen at  $-100^{\circ}\text{C}$ , a method favoured by Australian workers (Johnston, Pearson & Leatch, 1973).

In tropical countries reliable facilities may not exist for the supply of liquid nitrogen or solid carbon dioxide so that antigen may have to be stored at higher temperatures. This may apply also to antigen in transit between laboratories. Antigens of B. divergens and B. major (Joyner, Donnelly, Payne & Brocklesby, 1972) and of B. bigemina and B. berbera (Goldman, Pipano & Rosenberg, 1972) have been stored at  $-20^{\circ}\text{C}$ , but these authors do not discuss their choice of this temperature.

An experiment was set up in which both fixed and unfixed antigen slides of B. divergens were stored at four different temperatures by four normally available storage regimes. On four occasions after the preparation and storage of the antigen, a pair each of fixed and unfixed slides were used to titrate aliquots of a single positive serum sample. The effect of storage time at different temperatures on the titre of the positive serum is shown in Table 15. There was no difference between the fixed and unfixed antigen at any particular temperature and time, so the titres for each have been combined to give a geometric mean titre from four titrations at each temperature regime on each occasion.

Bearing in mind that only differences in excess of one doubling dilution are unlikely to occur by chance alone in repeat titrations, (see 'Reproducibility' later), Table 15 shows that all the methods of storage gave comparable results up to 26 days. This means that antigen slides could be sent by post between laboratories without deleterious effect. However, antigen stored at room temperature for 135 days was not titratable due to a high degree of non-specific fluorescence in both test and control cells which was not related to serum dilution. At 135 days, antigen stored at  $+4^{\circ}\text{C}$  gave reduced titres, but antigen stored at  $-20^{\circ}\text{C}$  appeared to be as durable as that stored at  $-70^{\circ}\text{C}$ .

In Nigeria antigen had to be kept at  $-20^{\circ}\text{C}$  and

Table 15

The effect of the time and temperature of antigen storage  
on I.F.A. titre

Storage	Storage temper- ature	Days of storage			
		2	4	26	135
Room	Ambient	1/10,240	1/7,250	1/10,240	Not tit- ratable
Refrig- erator	+4°C	1/14,450	1/10,240	1/7,250	1/3,550
Electric freezer	-20°C	1/10,240	1/10,240	1/14,450	1/7,250
'Dry ice'	-70°C	1/10,240	1/7,250	1/7,250	1/7,250

In the absence of specific comparative studies, it has been the most popular choice of fixative for *Parasita* spp. It has a practical advantage over aqueous fixative solutions in that it dries very quickly so that sera can be applied to slides immediately after fixation without the risk of over coagulating.

#### Optimise concentration

The brightness of fluorescence, both specific and, if it occurs, non-specific, is influenced by the concentration



although it was necessary to store it for longer than 135 days (final tests used antigen stored for 240 days), the results above indicate that the antigen was unlikely to have deteriorated.

#### Choice of fixative

The author followed the method of Latif (1972) for B. divergens and used acetone as a fixative. This was found to be very satisfactory for B. microti and B. bigemina as well. It is worth noting, however, that a variety of methods of fixation have been used for protozoan antigens in I.F.A. tests, and a standard fixative does not exist. Heat (for several Babesia spp., Ludford, 1969), formaldehyde (Latif, 1972, for trypanosomes), hydrochloric acid (Ross & Löhr, 1968, for B. bigemina) and no fixative at all (Johnston, Pearson & Leatch, 1973, for B. argentina) have all been employed in addition to acetone. Zwart, Perié, Keppler & Goedbloed (1973) compared several different fixatives for trypanosomes and found acetone to be the best. In the absence of specific comparative studies, it has been the most popular choice of fixative for Babesia spp. It has a practical advantage over aqueous fixative solutions in that it dries very quickly so that sera can be applied to slides immediately after fixation without the risk of sera coalescing.

#### Conjugate concentration

The brightness of fluorescence, both specific and, if it occurs, non-specific, is influenced by the concentration

of conjugate for any one dilution of serum. This is well illustrated by Plate 12 which shows the diminishing brightness of specific fluorescence of Babesia microti associated with increasing the dilution of conjugate from 1/10 to 1/200. Plate 12 also illustrates that the intensity of non-specific background fluorescence in negative control cells is related to conjugate concentration. Thus a measure of control over non-specific fluorescence is available by adjusting conjugate concentration. The reduction in brightness in turn affects the end-point in titrations. With B. divergens this effect was found to be slight. It amounted to a reduction in end-point of one doubling dilution over a range of conjugate dilutions from 1/10 to 1/200. With B. bigemina the effect was more marked. It is clear that for tests which are to be compared the selection and adoption of a standard dilution of conjugate is an important prerequisite.

For both B. divergens and B. bigemina work, the conjugate was titrated against known positive and negative sera in a chessboard pattern to establish the working conjugate dilution. This dilution was selected as one which gave good fluorescence with positive sera, minimal background fluorescence with negative sera and economic usage of the conjugate. A dilution of 1/100 was adopted.

#### Mounting preparations for viewing

It is customary to view preparations for fluorescence microscopy mounted under cover-slips in a suitable non-

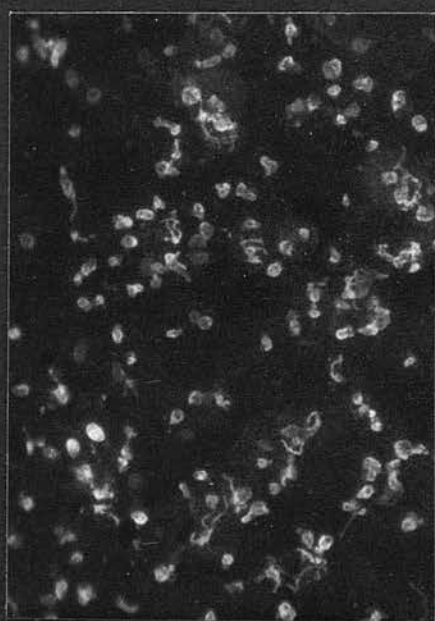
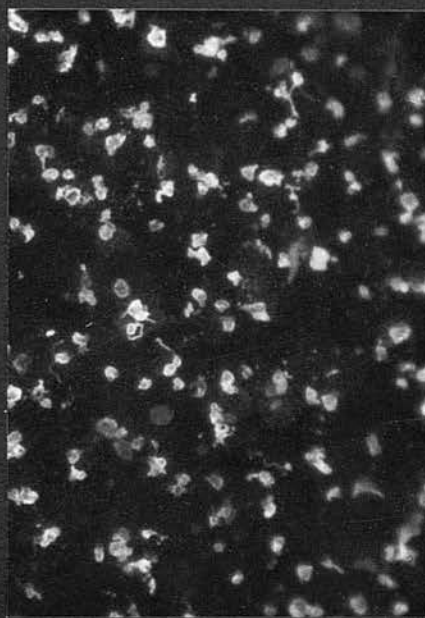
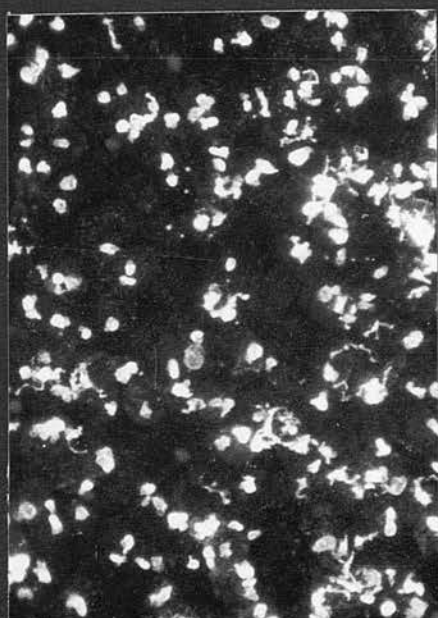
Plate 12

The effect of conjugate concentration on the intensity of fluorescence in the I.F.A. test (antigen B. microti).

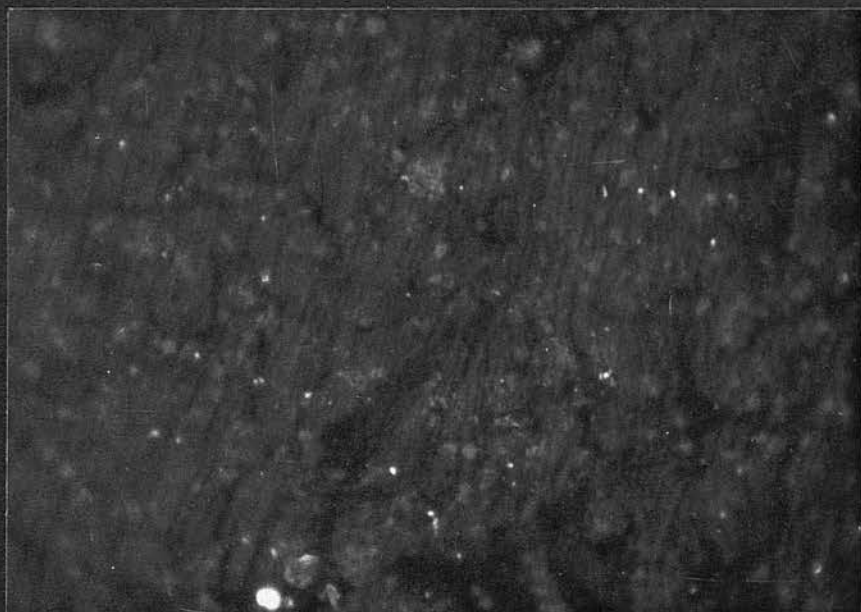
This page - positive serum at 1/40 dilution and conjugate dilutions of 1/10, 1/50, 1/100 and 1/200 (from top left to bottom right, respectively).

Next page - negative control serum at 1/40 dilution and conjugate dilutions of 1/10 (top) and 1/100 (bottom).

Constant photographic conditions were used throughout.







fluorescent medium such as buffered glycerol. This may provide a clearer field and allow preservation of the material, but was found to be of no advantage in the I.F.A. test for Babesia spp., when slides were always examined within minutes of the completion of the test. Mounting would be a difficult procedure in titrations, where a large number of cells are examined on a slide.

### The evaluation of the test

#### Reproducibility

During the course of many titrations in which duplicate slides were examined the agreement between duplicates for B. divergens was good i.e. over 90% of paired titrations agreed to within one doubling dilution. For replicate titrations, in which serum dilutions were also repeated, the experimental error was still small. For twenty-four titrations of twelve sera in which the repeat titration was carried out within 16 days the within pair difference did not exceed one doubling dilution. This means that a difference in titre of more than one doubling dilution is unlikely to be due to experimental error.

With B. bigemina, the test as used in Nigeria did not prove to be as reproducible as had been found with B. divergens. Even with fourfold dilution intervals, 50% of repeat titrations showed a disparity of between 1 and 2 dilution intervals. In sequential samples from single animals differences between repeat titrations were always



in the same direction; it was therefore the overall sensitivity of the test which apparently varied from day to day.

### Specificity

#### Incidence of false positives and false negatives

The test as applied to B. divergens proved to be highly specific. Of 26 cattle presumed to be unexposed to B. divergens, all were negative to the I.F.A. test, and of 14 cattle which recovered from B. divergens infection, all were positive.

The test as applied to B. bigemina was less reliable. It was highly specific using serum from indoor reared experimental calves, (9/9 negative before successful infection, 4/4 survivors positive), slightly less specific with serum from older sprayed cattle but still usable with confidence, but so poorly specific with serum from unsprayed Fulani cattle that it could not be used.

#### Cross-reactivity

Sera (kindly supplied by Dr. D.W. Brocklesby), from two cattle recovered from B. major infection, which reacted positively with homologous antigen (titres 1/640 and 1/160) did not react with B. divergens antigen, nor did high-titre (1/10,240) B. divergens antiserum react with B. major antigen.

Sera from animals recovered from incidental Theileria mutans or Eperythrozoon teganodes infection did not react with B. divergens antigen.

Because antigen of B. bovis was not available in Vom, cross-titrations were not carried out with B. bigemina.

#### Prozone effect

Fluorescence with positive serum at low dilutions was occasionally noticed to be less well pronounced than that obtained with medium dilutions of the same serum. Johnston, Pearson & Leatch (1973) have noted this effect with B. argentina, although at rather lower dilutions.

#### Non-specific staining

Unwanted staining which could not be eliminated by diluting conjugate was, on occasions, encountered for both B. divergens and B. bigemina. Its presence could be recognised by the fluorescence of parasites in negative serum control cells, and also, in most cases, by a different appearance of the non-specifically stained parasites.

Specifically stained parasites took on the conjugate uniformly around the periphery and a little less intensely in the centre (Plate 13). As the preparation faded during examination, the peripheral staining persisted longer than did the central staining. With paired B. divergens, this produced a highly characteristic 'spectacle-frame' appearance. This was not seen with non-specific staining. In the latter, parasites stained patchily.

With B. divergens, non-specific staining occurred only for a part of the time when the test was being used. In spite of exhaustive attempts to eliminate it (changing antigen, conjugate, reference sera, P.B.S. buffer, test

Plate 13

Babesia divergens - I.F.A. test, positive serum



method), it persisted only to disappear spontaneously. It was possibly due to a charge effect (Goldman, 1968) but the precise source of the trouble could not be identified.

The non-specific staining encountered with B. bigemina was different in character. It involved staining of red cells as well as parasites, and was related to the age of cattle tested and their exposure to other agents. It was probably essentially a degree of cross-reactivity. Serum from B. bovis infected cattle will cross-react with B. bigemina at low dilutions but not above dilutions of 1/160 (Ludford, 1969; Goldman, Pipano & Rosenberg, 1972; Leeftlang & Perié, 1972). In addition to true cross-reactions some of the unwanted fluorescence may have been caused by extraneous specific reactions between other antigens present in the Babesia bigemina infected smears, and their respective antibodies present in the serum of older cattle. The antigen was prepared from blood containing only B. bigemina at patent levels but the host calf was later shown at post-mortem to be infected with B. bovis. It is therefore possible that our I.F.A. antigen also contained antigens in some form, possibly as adsorbed soluble antigen, of this parasite. However, some unwanted fluorescence even occurred when serum from cattle newly imported from Britain, which had obviously never been infected with B. bovis, was tested. The precise cause of the unwanted fluorescence therefore remained undiscovered.

Whilst the presence of non-specific staining is

undesirable, it does not necessarily invalidate test results as long as control cells are included and sera are titrated. The I.F.A. test results for B. bigemina are presented with due consideration of the involvement of non-specific fluorescence.

Harvey (1935) reported this parasite as a contaminant with B. bigemina (Baker, 1934). It was not until 1950 that Harvey, Joyner & Kendall corroborated, with reference to the validity of B. bigemina, on the grounds of its morphological differences from B. bigemina.

B. bigemina infection is probably prevalent in cattle throughout each of the British Isles where Simulium exists, the vector (Joyner, Harvey & Kendall, 1951), is present. In some of these areas it is an important cause of disease (V.C.S.U. Report, 1959) though the recorded distribution and occurrence of cases probably falls far short of that which actually exists. In an interesting analysis of the situation, Bennett (1954) has deduced that there may be some 30,000 cases of babesiosis in Britain per year, which cost the livestock industry between £100,000 and £200,000 per annum. The impact of the disease is concentrated by its local distribution, so that in some areas (e.g. around Cork, in Ireland), babesiosis is the major summer disease of cattle.

The disease as it occurs in cattle in the British Isles has been described by Harvey (1935), Wright & Woodford (1938), Fallon (1949), Osborne (1952) and Collins,



B. Babesia divergens

Introduction

In 1911, M'Fadyean & Stockman described a new piroplasm of cattle in Britain which they called Piroplasma divergens. Subsequent authors (Wenyon, 1926; Neitz, 1956) regarded this parasite as synonymous with B. bovis (Babes, 1888). It was not until 1958 that Davies, Joyner & Kendall established, without doubt, the validity of B. divergens, on the grounds of its morphological difference from B. bovis.

B. divergens infection is probably prevalent in cattle throughout much of the British Isles where Ixodes ricinus, the vector (Joyner, Davies & Kendall, 1963), is present. In some of these areas it is an important cause of disease (V.C.O.U. Report, 1969) though the recorded distribution and occurrence of cases probably falls far short of that which actually occurs. In an interesting analysis of the situation, Barnett (1974) has deduced that there may be around 30,000 cases of babesiosis in Britain per year, which cost the livestock industry between £300,000 and £400,000 per annum. The impact of the disease is concentrated by its local distribution, so that in some areas (e.g. around Cork, in Ireland), babesiosis is the major summer disease of cattle.

The disease as it occurs in cattle in the British Isles has been described by Harvey (1935), Wright & Woodford (1958), Fallon (1965), Osborne (1970) and Collins,

O'Nualláin & Ferguson (1970). Descriptions of the laboratory infection of cattle have been given by Ryley (1957), Davies, Joyner & Kendall (1958), Lucas (1960), Joyner (1966), Joyner & Davies (1967) and Brocklesby, Harness & Sellwood (1971).

As with other Babesia spp., an inverted innate age resistance is said to be a feature of immunity to B. divergens, but Brocklesby, Harness & Sellwood (1971) failed to confirm this under laboratory conditions. Experimentally infected calves remain infected for several years and they remain immune to challenge even after parasites are no longer detectable (Joyner & Davies, 1967). No experimental studies of chronic parasitaemias or of the incidence of recrudescences have been conducted, but several authors comment that clinical relapses occur in naturally infected cattle after stress e.g. Richardson & Kendall (1963). In this connection, Harvey (1935) provides some interesting details. He opines that relapses may be provoked by oestrus, storms, parturition, over-driving, transit and sudden changes (e.g. climatic extremes) but apparently not by acute febrile or debilitating conditions such as emaciation, over-crowding, other parasites and deficiency disease.

Serological studies of B. divergens have been principally concerned with the use of the I.F.A. test to differentiate the organism from B. major (Joyner, Donnelly, Payne & Brocklesby, 1972) and other Babesia

spp. (Leefflang & Perié, 1972). The I.F.A. test has also been applied in the field to determine the incidence of B. divergens in an infected herd (Donnelly, Joyner & Crossman, 1972) and in an endemic area (Latif & Wells, 1973). A serological survey using the I.F.A. test is currently being conducted in Scottish cattle, and this could yield some very interesting epidemiological information (D.A. Blewett, pers. comm.). Information on the basic I.F.A. response in cattle is lacking.

Observations on the primary infection of cattle with  
B. divergens

a) Splenectomised calves

Materials and methods

Nine splenectomised cattle were infected with B. divergens in three experiments. Details of the cattle and the inocula are given in Table 16.

Cattle were examined and bled daily for 24 days after infection and less frequently thereafter. As well as percentage parasitaemias, absolute parasitaemias\* were also calculated.

Data from all nine cattle are included for analysis of the time course of reactions since these can be related to the day of maximum parasitaemia. For measurements of magnitude, mean values have been calculated only from the homogeneous group of seven cattle which were infected simultaneously with the same dose of parasites. Values

\* - see Appendix 2

## Results

Table 16

Details of splenectomised cattle and their inocula involved in B. divergens experiments

Experiment	Animal number	Age (months)	Inoculum*	Source
B.d.1	10	5	About $10^7$	TREU 1081
B.d.2	212, 273, 274, 275, A89, EC251 and L1160	$4\frac{1}{2}$	$5.0 \times 10^8$	TREU 1155
B.d.3	296	13	$5.0 \times 10^8$	TREU 1155

\* - Parasitised erythrocytes/100 kgms. weight (the parasite density in stabulates was established before freezing).

Death in two animals occurred two days after maximum parasitaemia, following a brief period of illness.

Another calf (272) became seriously ill, exhibited symptoms of pulmonary oedema and remained 'down' for over a week. With vigorous supportive treatment it recovered. Specific bactericidal drugs were not given.

\* - see Appendix 2

for the other two cattle infected were very similar.

### Results

All cattle became infected, developed moderate to high parasitaemias and suffered varying degrees of illness. Two animals died. The characteristics of infection in the three groups of cattle were very similar.

#### (1) Clinical symptoms

The clinical effects are summarised in Table 17 which shows the proportion of animals exhibiting a particular symptom.

Table 17

The occurrence of clinical symptoms following B. divergens infection in nine splenectomised steers

Fever*	Haemoglob- inuria	Anorexia	Diarrhoea	Jaundice	Death
8/9	6/9	3/9	1/9	1/9	2/9

As a general rule, the severity of illness and the number of symptoms exhibited was positively correlated with the magnitude of the maximum parasitaemia.

Death in two animals occurred two days after maximum parasitaemia, following a brief period of illness. Another calf (212) became seriously ill, exhibited symptoms of pulmonary oedema and remained 'down' for over a week. With vigorous supportive treatment it recovered. Specific babesicidal drugs were not given.

\* - see Appendix 2



(ii) The parasite

The morphology of the parasite was as described by Davies, Joyner & Kendall (1958). With acridine orange staining, the cytoplasm stained a uniform orange which frequently masked the yellow-green stained nucleus. Parasites were usually peripheral and frequently appeared to extend beyond the erythrocyte margin (Plate 7). Although divergent pairs of organisms are characteristic of B. divergens, about 90% of parasitised erythrocytes contained only one organism. The incidence of paired organisms was determined at different stages of parasitaemia in each of six calves. There was no difference between the incidence of paired organisms either when the parasitaemia was rising rapidly, held stationary or declining rapidly. A very few erythrocytes, each of which contained three parasites, were observed, but multiple parasitism beyond this level was not seen.

At the height of parasitaemia and just afterwards, Giemsa-stained thin smears were examined for signs of the 'droplet' form of organism as seen with B. rodhaini in rats. This was not observed. Certain abnormal forms were evident after peak parasitaemia. These were smaller than usual, and consisted of a pink chromatin mass and a much reduced densely-stained mass of cytoplasm. Later in the course of infection, surviving parasites did not differ in morphology from forms present during the rising parasitaemia.



During the recovery phase, when immature erythrocytes were present in smears, parasites were seen in both 'stippled' cells and reticulocytes but there was no noticeable preference for such cells.

(iii) Parasitaemia

Some parameters of the parasitaemias, together with parameters of fever, are presented in Table 18.

At later stages in infection, when animals became anaemic, the percentage parasitaemia sometimes gave a false estimate of the state of parasitaemia. For this reason, most of the following analysis utilises values for absolute parasitaemia rather than for percentage parasitaemia.

It can be seen from Table 18 that the individual parasitaemias paralleled one another in development but were slightly out of phase. By synchronising parasitaemias to the day of maximum parasitaemia, the growth curve in Fig. 12 was obtained, which shows the mean absolute parasitaemias for the group of seven. Note that by the process of synchronisation the standard deviations have been reduced to small values.

Maximum parasitaemias were reached 3-6 ( $4.0 \pm 0.7$ ) days after patency. Thereafter, the parasitaemia remained patent for a variable length of time. During this stage, a lesser, second peak of parasitaemia occurred in five out of seven surviving cattle. This phenomenon is partially obscured by averaging the data, as in Fig. 12, since

Table 18

Some parameters of parasitaemia and fever in splenectomised cattle  
infected with B. divergens

	10	296	212	273	274	275	A89	EC251	L1160
Prepatent period (days)	6	6	8	7	7	7	7	7	8
Day of maximum absolute parasitaemia	10	10	12	12	10	11	10	11	13
Duration of parasitaemia (days)	8	d.12*	14	9	16	16	d.12*	9	14
First day of fever	7	9	11	-	10	10	10	10	12
Day of maximum fever	10	9	13	-	10	12	10	11	13
Duration of fever (days)	8	3	3	-	1	5	2	8	4

\* - Day of death

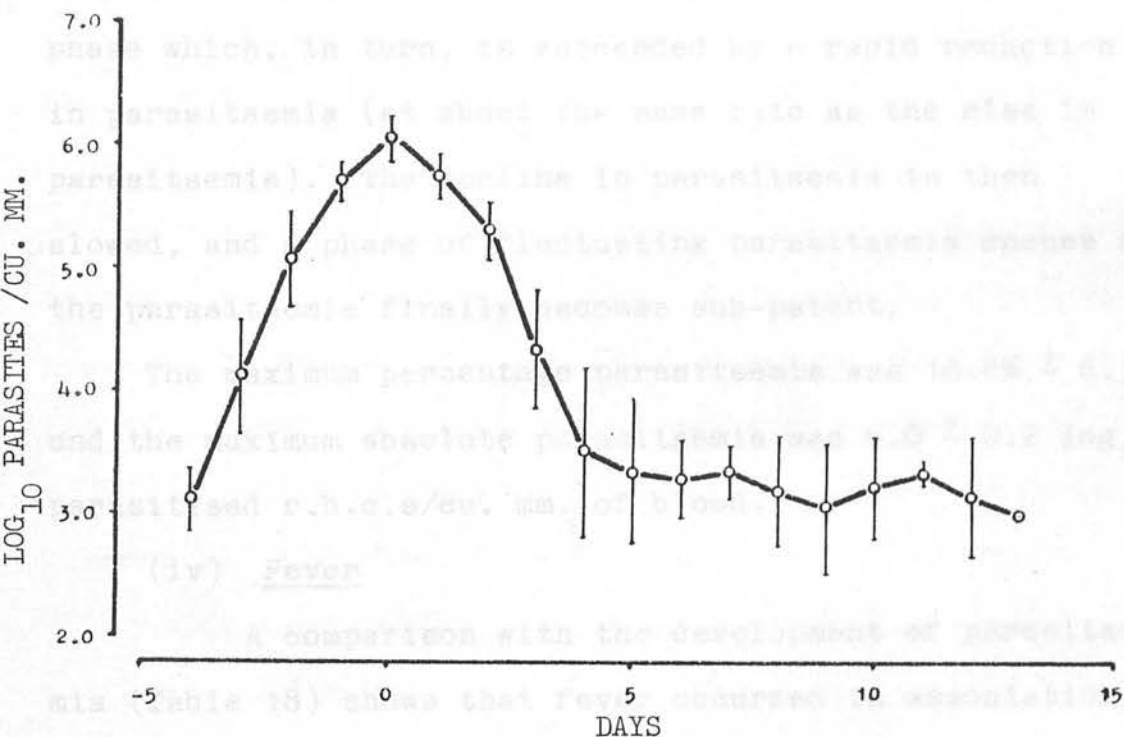


Fig. 12

The parasitaemia curve of B. divergens.

Individual absolute parasitaemias for seven cattle were synchronised to the day of maximum parasitaemia (day 0) and then averaged. Vertical bars denote standard deviations of the means. (From day +3 the mean is derived from fewer than 7 animals).

parasitaemias at this stage had drifted out of synchrony. The interval between peak primary parasitaemia and peak secondary parasitaemia was between 8-13 ( $10.2 \pm 2.2$ ) days.

The parasitaemia curve shown in Fig. 12 consists of four phases. A phase of logarithmic growth (which, measured graphically, amounts to almost exactly a tenfold increase in parasitaemia per day), is followed by a 'lag' phase which, in turn, is succeeded by a rapid reduction in parasitaemia (at about the same rate as the rise in parasitaemia). The decline in parasitaemia is then slowed, and a phase of fluctuating parasitaemia ensues as the parasitaemia finally becomes sub-patent.

The maximum percentage parasitaemia was  $18.8\% \pm 8.3\%$  and the maximum absolute parasitaemia was  $6.0 \pm 0.2 \log_{10}$  parasitised r.b.c.s/cu. mm. of blood.

(iv) Fever

A comparison with the development of parasitaemia (Table 18) shows that fever occurred in association with higher parasitaemias (but was not present for the first days of patency) and that maximum fever (the mean of which was  $40.3^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ ) coincided with maximum parasitaemias.

In terms of their duration and magnitude, there was no correlation between fever and parasitaemia.

(v) Anaemia

Pre-infection levels of P.C.V. ( $33.7\% \pm 3.1\%$ ) were maintained up to the day of maximum parasitaemia.

On this day a sharp decline in P.C.V. occurred (Fig. 13). The decline in P.C.V. reached a nadir ( $9.2\% \pm 2.3\%$ ) two to five ( $3.1 \pm 0.9$ ) days after maximum absolute parasitaemia.

Haemoglobinuria was first seen on or about the day of maximum parasitaemia when P.C.V. first declined. Immature erythrocytes (normoblasts, polychromatophils and 'stippled' cells) appeared in surviving cattle one to five days after maximum parasitaemia, coincident with the nadir in P.C.V.

For the group of seven, the maximum depression in P.C.V. was  $73\% \pm 5\%$  of pre-infection levels. In the seven cattle which survived infection, there was a positive correlation between the maximum percentage parasitaemia and the maximum percentage depression in P.C.V. ( $r = +0.85$ ,  $p = < 0.05$ , significant). There was no correlation between the duration of parasitaemia and the maximum percentage depression in P.C.V. ( $r = 0.37$ ,  $p = > 0.05$ ).

(vi) Leucocytes

In all animals, a leucopenia preceded maximum parasitaemia by two to four ( $2.5 \pm 0.7$ ) days. Total W.B.C. counts then rose rapidly to a peak in excess of pre-infection levels, one to three ( $2.1 \pm 0.8$ ) days after peak parasitaemia. In six out of seven surviving cattle, a second, lesser nadir followed at a more variable time, ( $6.0 \pm 1.4$  days) after peak parasitaemia. Thereafter, W.B.C. counts slowly returned to pre-infection levels.

For the homogenous group of seven, mean values

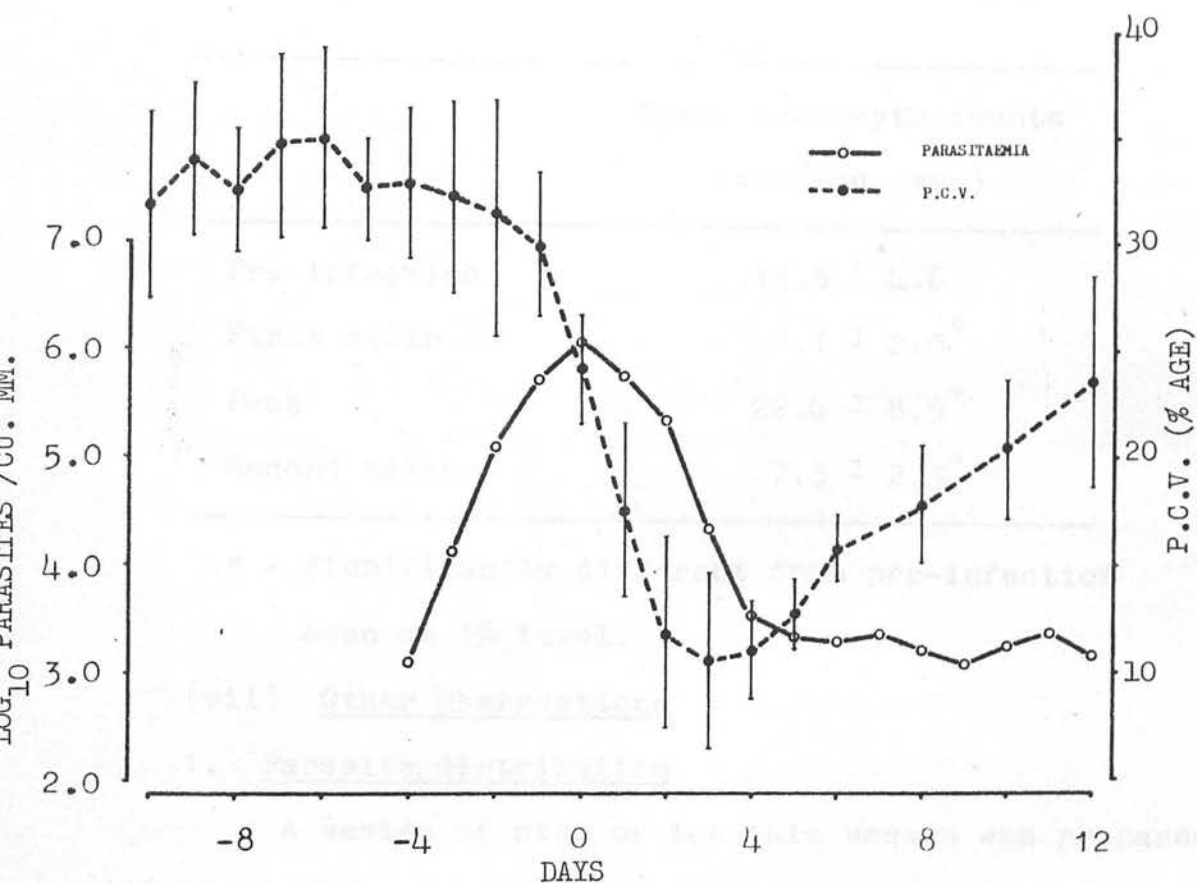


Fig. 13

The decline in P.C.V. related to the parasitaemia in B. divergens infected splenectomised calves. (Daily values for P.C.V. have been synchronised to the day of maximum parasitaemia - day 0 - and then averaged; vertical bars denote standard deviations of the mean).



are presented in Table 19 and compared statistically with the pre-infection group mean.

Table 19

Leucocyte counts during B. divergens infection of splenectomised calves

	Total leucocyte counts ( $\times 10^3$ /cu. mm.)
Pre-infection	$13.5 \pm 4.6$
First nadir	$7.1 \pm 2.0^*$
Peak	$22.4 \pm 8.9^*$
Second nadir	$7.3 \pm 2.3^*$

\* - Significantly different from pre-infection mean at 1% level.

(vii) Other observations

1. Parasite distribution

A series of nine or ten thin smears was prepared with blood drawn from each of four potential sampling sites from a steer with a patent B. divergens parasitaemia. In this way, a mean local parasitaemia was derived for each site and these means were then compared. The experiment was repeated.

Blood was collected from the jugular and coccygeal veins by venipuncture with needle and 'Vacutainer' and from a superficial ear vein and the tail tip by 'pricking' a cleaned and clipped area of skin.

The results of the two experiments are shown in Table 20.

Table 20

Mean B. divergens parasitaemias in blood from different sampling sites  
(two experiments)

Jugular vein	Coccygeal vein	Ear vein	Tail tip
16.2% $\pm$ 1.2%	16.5% $\pm$ 2.1%	14.4% $\pm$ 2.4%	16.5% $\pm$ 5.0%
3.5% $\pm$ 1.0%	3.8% $\pm$ 0.7%	4.0% $\pm$ 1.1%	3.8% $\pm$ 0.6%

No statistically significant difference occurred between parasitaemias at any of the sampling sites ( $p = > 0.05$ ).

Parasitaemias in internal organs were determined, post-mortem, in one calf which died from babesiosis. One smear only was prepared from each site, so that the 'within site' variance cannot be calculated. The parasitaemias were as follows:- cerebral vein 22%, jugular vein 21%, and in impression smears, kidney 12%, heart 19%, liver 31%. It is apparent that very marked differences do not exist.

## 2. Attempted concentration of B. divergens parasitised erythrocytes by osmotic lysis

A schizont-infected cell agglutination test for Plasmodium knowlesi has been shown to be variant specific (Brown, Brown & Hills, 1968). A similar test using

piroplasm-infected erythrocytes was adapted by Curnow (1968) to demonstrate antigenic variation between strains of B. argentina. To produce an 80% infected red cell suspension, Curnow (1968) concentrated low parasitaemias by the method of Mahoney (1967). This method exploits the increased resistance to lysis of B. argentina infected erythrocytes compared with non-infected erythrocytes. Using saline at 4.2 gms. sodium chloride/litre, Mahoney (1967) obtained optimum concentration of B. argentina infected cells. It was decided to apply this concentration method to B. divergens, in view of its possible usefulness in allowing the preparation of antigen from low parasitaemias, and its potential as an aid in detecting low grade parasitaemias.

A thin smear was made from a sample of well-mixed B. divergens parasitised blood. Aliquots of 0.5 ml. of blood were then dispensed into four tubes. The tubes were centrifuged, the plasma was discarded and 4.5 mls. of saline were added to each tube at the following concentrations - 3, 4, 5 and 6 gms./litre. The tubes were allowed to stand at room temperature for 15 minutes and were then spun at 1500 r.p.m. (600 g) for 10 minutes. The supernatants were poured off, the cells were resuspended in saline, and thin smears were prepared from this suspension. All smears were stained by Giemsa's method.

After centrifugation, the four tubes held different amounts of sediment. The greatest amount was in the

6 gms./litre tube, and the least amount was in the 3 gms./litre tube. The supernatant in each tube was red in colour. Parasitaemias from whole blood and from the post-saline treated cell suspensions are shown in Table 21. It is apparent that infected cells were not concentrated by this means; indeed it appeared that they were more easily lysed than non-infected erythrocytes.

Table 21

The effect of different concentrations of saline on

B. divergens infected blood

Treatment	Parasitaemia	Notes
Untreated blood	17.5%	
Saline treated @ 3 gms./litre	-	No intact r.b.c.s. Abundant free parasites.
@ 4 gms./litre	-	Abundant free parasites. Very few intact r.b.c.s and majority of these not parasitised.
@ 5 gms./litre	0.5%	Many intact r.b.c.s but very few parasitised. Some free parasites.
@ 6 gms./litre	7.5%	Very few free parasites.

b) Non-splenectomised cattle

Materials and methods

Four cattle were infected with stabulates of B. divergens in two experiments. Details of the cattle are given in Table 22. Cattle were examined and bled daily for 24 days after infection.

Table 22

Details of non-splenectomised cattle and their inocula  
involved in B. divergens experiments

Experiment	Animal number	Age (months)	Inoculum*	Source
B.d.(a)	1097	15	$1.6 \times 10^9$	Stabilate
B.d.5	270	36	$5.0 \times 10^8$	TREU 1155
	451	36	$5.0 \times 10^8$	TREU 1155
	452	36	$5.0 \times 10^8$	TREU 1155

\* - Parasitised erythrocytes/100 kgms. weight

Table 23

Parameters of parasitaemias in B. divergens infected  
non-splenectomised steers

	1097	270	451	452
Prepatent period (days)	5	6	8	7
Day of maximum parasitaemia	5	6	11	10
Duration of parasitaemia (days)	2	1	4	5
Maximum parasitaemia %	0.020	0.001	0.023	0.034

## Results

None of the cattle was clinically affected and all recovered uneventfully. A low and transient parasitaemia was observed in all of them. Some parameters of the parasitaemias are shown in Table 23. In two animals a second, lower parasitaemia was observed seven days after the maximum primary parasitaemia.

Changes in P.C.V. and total W.B.C. count did not occur in association with the parasitaemias, except for a slight leucopenia in one animal.

For the group of three, the maximum percentage parasitaemia was  $0.019\% \pm 0.17\%$  and the maximum absolute parasitaemia was  $2.9 \pm 0.9 \log_{10}$  parasitised cells/cu. mm.

### c) The Indirect Fluorescent Antibody response

#### Materials and methods

Splenectomised calves: sera, collected on days 10, 12, 14, 16, 20, 24, 31, 35, 45, 55 and 135 after infection, were titrated from six of the seven calves infected in experiment B.d.2 (see earlier section for details of these cattle and their reactions).

Non-splenectomised cattle: sera, collected on days 7, 10, 13, 16, 19, 25, 30, 35, 46, 60 and 99 after infection, were titrated from the three cattle infected in experiment B.d.5 (see previous section for details of these cattle and their reactions).

All the sera were titrated at twofold dilution intervals against B. divergens antigen.



## Results

### Splenectomised calves

The results of antibody titrations for five splenectomised calves which recovered from infection are represented in Fig. 14. In no case was antibody detected before maximum parasitaemia. Antibody levels rose quickly to near maximum levels, although maximum titres were usually not reached until some thirty-five days after infection. A sixth calf died twelve days after infection, two days after maximum parasitaemia. Antibody was not detected in its serum on the day of death.

### Non-splenectomised cattle

In non-splenectomised cattle the pattern of antibody response was similar to that of the splenectomised calves. The animal with the shortest prepatent period was the first to show antibodies, which in all three animals appeared on, or just after, the day of maximum parasitaemia. Antibody levels rose sharply within the next six days, whereupon titres stabilised at, or near, maximum levels. Table 24 compares some parameters of the antibody response of the splenectomised and non-splenectomised cattle.

Whilst the two groups differed significantly in maximum parasitaemia ( $p = < 0.01$ ), there was no significant difference between the maximum titres ( $p = > 0.05$ ).

In both groups a relatively sharp fall in titre after maximum levels was followed by a much slower

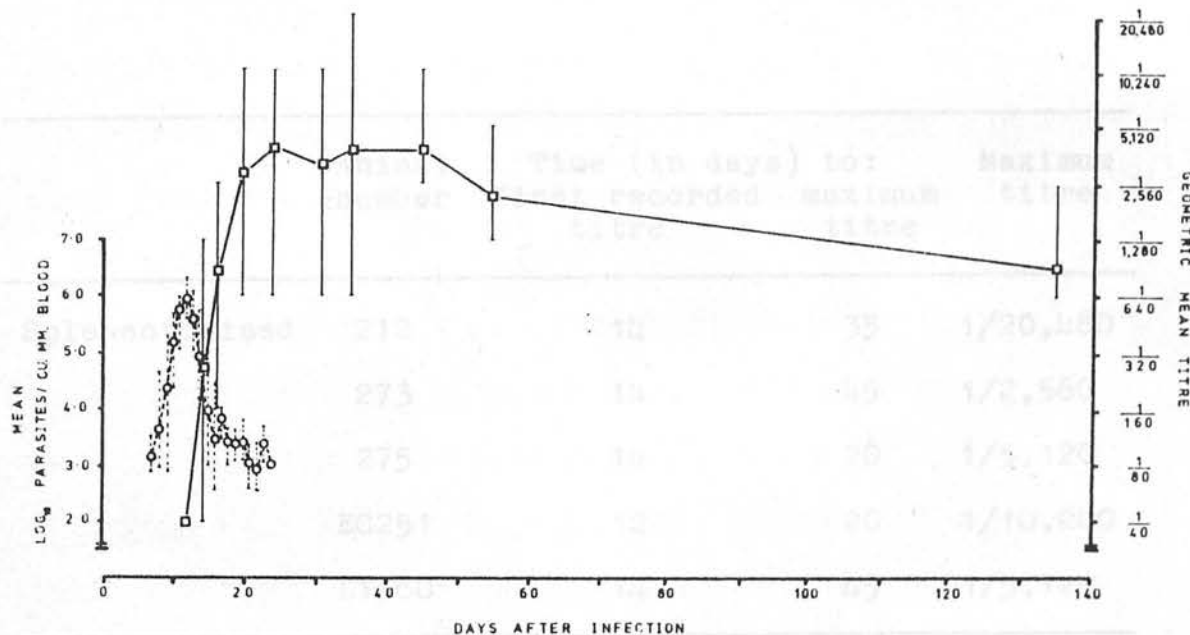


Fig. 14

Parasitaemia (dotted line) and I.F.A. titre (solid line) in splenectomised calves infected with B. divergens.

Vertical lines show ranges about the means.

Table 24

Some parameters of the I.F.A. response to B. divergens  
infection of splenectomised calves and  
non-splenectomised cattle

	Animal number	Time (in days) to: first recorded titre	to: maximum titre	Maximum titre
Splenectomised	212	14	35	1/20,480
	273	14	45	1/2,560
	275	14	20	1/5,120
	EC251	12	20	1/10,240
	L1160	14	45	1/5,120
Non- splenectomised	270	7	10	1/1,280
	451	13	25	1/10,240
	452	10	16	1/2,560

decline, and titres persisted for many months. The persistence of titres is illustrated in Fig. 15. Data for an additional six cattle, each of which had recovered from a single laboratory induced B. divergens infection, are also included. None of the cattle, to which the titres in Fig. 15 relate, had been involved in experiments, or handled, between the period immediately following its infection and the time of serum sampling.

At the time of sampling, all the splenectomised cattle included in Fig. 15 were patently infected at low levels. The non-splenectomised cattle were not patently infected. It was not known if they were still latently infected.

### Discussion

It is a well known protozoological fact that the susceptibility of an animal to infection with a Babesia spp. can usually be increased by removing the spleen. B. divergens has been shown previously (Davies, Joyner & Kendall, 1958), and in the present study, to be no exception to this rule. Parasitaemias were much greater and clinical reactions far more severe in splenectomised than in non-splenectomised cattle.

Only very low parasitaemias occurred in non-splenectomised adults and these were not associated with pathological changes. Similar results were obtained by Brocklesby, Harness & Sellwood (1971), who accounted for the mild infections experienced, by suggesting that the maintenance of Babesia spp. by syringe passage possibly



emasculated the parasite. The suggestion would seem to be supported by the reports of Ryley (1957) and Davies, Joyner & Kendall (1958), each of whom produced clinical illness in non-splenectomised adult cattle by inoculating blood recently isolated from field cases.

The relative avirulence of the Weybridge strain in our experiments was also apparent in infections of splenectomised calves, in comparison with the results of Ryley (1957). He observed haemoglobinuria in each of seven infected calves, five of which died. In the present studies 'redwater' was seen in 6/9 calves, and only two died. Using the Weybridge strain, Lucas (1960) reported similar results i.e. 5/6 calves with 'redwater' and one death only (an interesting indication of how quickly the Weybridge strain lost virulence). It is fair to point out, however, that comparisons between the results of early experiments and our own must be drawn with care, since the number of parasites inoculated was imprecisely known in most of the early work. Davies, Joyner & Kendall (1958) presented evidence suggesting that inoculum size influences the severity of disease and, furthermore, Ryley (1957) deliberately increased the virulence of his parasite by rapid passage.

In splenectomised calves moderately high parasitaemias occurred, with, in most cases, a clinical reaction. Fever was the symptom observed most frequently. 'Pipe-stem' diarrhoea - a classic symptom of babesiosis - was



not observed, possibly due to the dry nature of the feed given to the experimental calves.

The sequence in which parasites and symptoms appeared was as follows - parasites, fever, haemoglobinuria, anaemia. Generally speaking, symptoms correlated, in magnitude and timing, with parasitaemia. Maximum pyrexia coincided, to within a day, with maximum parasitaemia, but, in common with Davies, Joyner & Kendall (1958), no other correlations between parameters of fever and parasitaemia were significant.

The pattern of parasitaemia determined in the present study, resembles that for some individual calves given by Davies, Joyner & Kendall (1958). These workers also observed recrudescent parasitaemias, which were approximately synchronous, in four splenectomised calves. The relapse interval, estimated from Fig. 4 of their paper, ranged from 20-27 days. Working with a strain of B. divergens isolated in Somerset, Brocklesby, Harness & Sellwood (1971) observed a tenfold increase in parasitaemia/day in splenectomised calves. These calves suffered a recrudescent parasitaemia with a relapse interval of 10 days. A relapse parasitaemia was also evident in non-splenectomised cows. Data from Lucas (1960) also show a recrudescent parasitaemia in two splenectomised calves 4 and 5 days after peak primary parasitaemia. It is apparent that a secondary parasitaemia is a frequent feature of B. divergens infections in splenectomised calves, and

our own observations and those of Brocklesby, Harness & Sellwood (1971), show that it also occurs in non-splenectomised cattle. The growth characteristics of the strain of B. divergens used in our own studies (Weybridge strain) were very similar to those of the strain used by Brocklesby, Harness & Sellwood (1971).

In infections of splenectomised calves, the degree of anaemia was correlated significantly with the magnitude of parasitaemia. This correlation would best be shown if a wide range of parasitaemias was produced, perhaps by titrating the inoculum dosage. This is what Davies, Joyner & Kendall (1958) did and, although they do not make statistical correlations, their data show a marked negative correlation between minimum P.C.V. and maximum parasitaemia.

In our infections of splenectomised calves, P.C.V.s did not decline until parasitaemia was at or near maximum levels. Moreover, immature erythrocytes were not observed until several days later. This simple observation has some profound implications. It means that during the growth phase, whilst parasitaemia was increasing tenfold per day, no significant amount of red cell destruction occurred. It was not until the growth rate of parasitaemia had been arrested that massive haemolysis occurred, causing a sudden decline in P.C.V. and the appearance of haemoglobinuria. It appears, therefore, that haemolysis was associated with the host defence response and with

the destruction of parasites. The role of such factors in the pathogenesis of Babesia spp. infections has been known for some time because of various observations e.g. the disproportionately high degree of red cell destruction which accompanies low parasitaemias of certain species (Nissle, 1905), the pathological sequelae to massive parasite destruction by drugs (Sergeant, Donatien, Parrot, Lestoquard & Plantureux, 1927) and the persistence of haemolysis in B. canis infections after the decline in parasitaemia (Neitz, 1938). A variety of mechanisms including auto-immune phenomena, the production of lysins and toxins and hyperactivity of the R.E. system are probably involved (see Mahoney, 1972). A close examination of data presented by Lucas (1960) and Brocklesby, Harness & Sellwood (1971) for splenectomised calves infected with B. divergens, revealed the same pattern of haemolysis associated with maximum parasitaemia, with minimal red cell destruction beforehand.

The leucocytic response to Babesia spp. infections appears to be very variable. It is influenced by such factors as the presence or absence of a spleen and the fate of the host. The only feature of any constancy in the literature is that a lymphocytosis usually occurs (Maegraith, Gilles & Devakul, 1957; Wright, 1973; Gamble, 1974 and others).

The parasitaemia was shown not to vary between four sampling sites. This has the practical implication that

no site is advantageous for the detection of low parasitaemias. It has also been shown that low parasitaemias cannot be concentrated in vitro by lysis of infected blood in hypotonic saline. This fact is in agreement with the findings of Dolan (1974) that the osmotic fragility of B. divergens infected blood is greater than that of non-infected blood.

There was no striking difference between the young splenectomised and the adult non-splenectomised cattle in the magnitude or the time course of the antibody response. Closer scrutiny of the data suggests that the non-splenectomised cattle were slightly quicker in producing antibodies as judged by a comparison of the interval between first day of patency of parasitaemia and first appearance of antibodies. Serum samples were not, however, taken frequently enough at the critical phase to confirm the existence of this difference.

In both groups, the antibody rise correlated with the fall in parasitaemia rather than with its rise. This contrasts markedly with the response of mice to Babesia microti infections described by Cox & Turner (1970), in which both IgM and IgG levels were elevated before peak parasitaemia was reached. Cox & Turner (1970) did not find that IgM appeared any earlier than IgG, but Mahoney (1972) has produced evidence that, in cattle infected with B. argentina, a shift from IgM to IgG synthesis takes place over a protracted period. In infections with

some piroplasms it has been established that IgM precedes IgG in appearance (Takahashi, Yamashita, Isayama & Shimizu, 1976). Since antibody measured in the present instance was IgG, it is possible that this fact is responsible for the apparently late appearance of antibody. As in the case of B. microti infections of mice, antibody levels to B. divergens continued to rise after the parasitaemia had fallen to near sub-patent levels. In some animals there was a slight diphasic response with the second peak being the maximum, and this might be related to the occurrence of secondary (first relapse) parasitaemias. Data given by Joyner, Donnelly, Payne & Brocklesby (1972) show a similar diphasic response in a calf infected with B. major.

A feature of the antibody response was that animals were serologically negative during the early course of clinical disease. After a parasitaemia lasting five days, one splenectomised animal died without antibodies being detected in its serum.

Antibody levels declined slowly after maximum levels had been reached and significant titres were recorded for at least 500 days. In all the splenectomised animals, a positive titre was associated with the persistence of infection. This accords with the behaviour of I.F.A. titres in other Babesia spp. infections (Ross & Löhr, 1968; Johnston & Tammemagi, 1969; Leeftang & Perié, 1972).

Although the spleen is a major site of antibody



formation against blood parasites (Taliaferro, 1955), it is apparent from the behaviour of the splenectomised cattle that their ability to produce antibody as measured by the I.F.A. test was not greatly impaired by the absence of a spleen. Similarly Wolf (1974) has reported that splenectomised hamsters infected with B. microti exhibit an I.F.A. response comparable to that of non-splenectomised hamsters. Garnham & Voller (1965) showed that splenectomised monkeys produced I.F. antibodies against B. divergens, though no comparison with non-splenectomised animals was possible, since these proved refractory to infection with the organism. However, Taliaferro (1955), and more recently Todorovic, Ferris & Ristic (1967), have drawn attention to the fact that the spleen is the first site of antibody synthesis to respond to blood-borne antigen. Todorovic, Ferris & Ristic (1967) found that, compared with intact rats, the development of agglutinins was delayed by six days in splenectomised rats infected with B. rodhaini, although prepatent periods were similar. Moreover, peak agglutinin titres were lower in the splenectomised rats although parasitaemias, and presumably levels of antigenic challenge, were higher and more prolonged. In B. divergens infections, splenectomised cattle also suffered much higher and more prolonged parasitaemias than non-splenectomised animals, but in contrast to the situation in rats, splenectomised calves produced as much, if not more antibody as measured by the I.F.A. test, as



non-splenectomised cattle. Although the absence of a spleen may have delayed the production of I.F. antibody, it did so by a relatively short time. This suggests that either extra-splenic sites of antibody formation respond more effectively to antigenic challenge in the case of B. divergens infecting cattle than in the case of B. rodhaini infecting rats, or that the production of agglutinins is more severely affected by splenectomy than is the production of I.F. antibodies. It is relevant to note that Bruce-Chwatt, Dorrell & Topley (1972) found that with mice infected with Plasmodium berghei yoelli, splenectomy increased the mortality rate, but in those splenectomised mice which survived, the production of I.F. antibodies was unimpaired.

A base dilution of 1/40 was chosen for the conduct of the test in order to eliminate interference by non-specific fluorescence. A prozone-like effect was occasionally noted even at this dilution. Johnston, Pearson & Leatch (1973) have also observed this phenomenon, though at rather lower dilutions. This is another reason for commencing titrations and conducting screening tests at a moderately high dilution of serum.

#### Observations on chronic parasitaemias and some factors which might influence them

##### a) A pilot experiment

This was conducted in order to find out if recrudescences of parasitaemia, detectable by the examination of

acridine orange stained thin blood smears, could be provoked in either splenectomised or non-splenectomised cattle by B-methasone administration. The results would indicate the choice of animal and the method of assessing parasitaemia which would be suitable for future experimentation.

Blood glucose estimations were made in order to confirm the activity of the B-methasone.

#### Materials and methods

Five steers of between 12 and 20 months of age, which had recovered from a single laboratory induced infection of B. divergens, were divided into a treated group and a non-treated infected control as shown in Table 25. In addition, a 20 month old, non-infected steer was subjected to B-methasone treatment as a treated non-infected control.

Treated cattle were injected intramuscularly with B-methasone ('Betsolan', Glaxo, Greenford, Middlesex) at 0.1 mgm./kgm. weight/day for five days, beginning on day 0.

All cattle were examined, and thin blood smears were prepared, daily from day 0 to day 18, and on days -3 and -5. The cattle were also bled for a second time (four hours post-treatment) on day 0.

Haematological examinations (P.C.V., W.B.C. and R.B.C. estimations and differential counts) were performed at frequent intervals before, during and after treatment.

Blood glucose was determined by the glucose oxidase

method on deproteinised blood using a 'Biochemica' test kit (Boehringer Corporation Ltd., Ealing, London).

On one occasion after treatment, faecal samples were examined for coccidial oocysts.

### Results

The results are summarised in Table 25. Recrudescient parasitaemias of 0.021% and 0.008% occurred on day 4 only.

One splenectomised animal developed severe clinical coccidiosis after B-methasone treatment and was destroyed on day 11. On the same day, oocyst counts on faecal samples from the other five cattle were low (50-550 oocysts/gm.). None of these cattle suffered any illness following B-methasone treatment.

The red cell picture was not markedly affected by the administration of B-methasone, but very significant leucocytic changes did occur. These are illustrated in Table 26 and Fig. 16. The principal effect of treatment was a lymphocytosis which comprised a massive neutrophilia, a lymphocytopenia (which led to a crossover in the lymphocyte/neutrophil ratio) and an eosinopenia.

The mean blood glucose concentration for the five treated animals before treatment was  $56.4 \pm 3.0$  mgms./100 mls. Blood glucose levels were significantly elevated within four hours of the first injection of corticosteroid. Levels maximised on day 1 ( $89.0 \pm 9.7$  mgms./100 mls.), remained elevated during treatment and returned

Table 25

The occurrence of recrudescent parasitaemias in B. divergens  
recovered cattle following B-methasone administration

			Months since infection	Recrudescent parasitaemia
Treated	Splenectomised	X1	5	+ve
		X3	14	+ve
	Non-splenectomised	1095	5	-ve
		1097	4	-ve
Non-treated	Non-splenectomised	1094	2	-ve

Table 26

Total leucocyte and absolute differential counts in five cattle before and after B-methasone treatment  
(counts  $\times 10^3/\text{cu. mm.}$ )

	Total leucocytes	Lymphocytes	Neutrophils
Pre-treatment	$11.2 \pm 2.0$	$8.3 \pm 1.5$	$1.8 \pm 0.4$
	Sig. 5%	Sig. 1%	Sig. 1%
Post-treatment extremes	$17.5 \pm 3.9$	$3.4 \pm 0.6$	$12.5 \pm 3.6$

(The pre- and post-treatment counts have been compared statistically and the level of significant difference is indicated in this and subsequent similar tables)

Fig. 15

The effect of B-methasone on absolute  
differential leucocyte counts  
(Vertical bars show standard error  
of the means from five subjects)

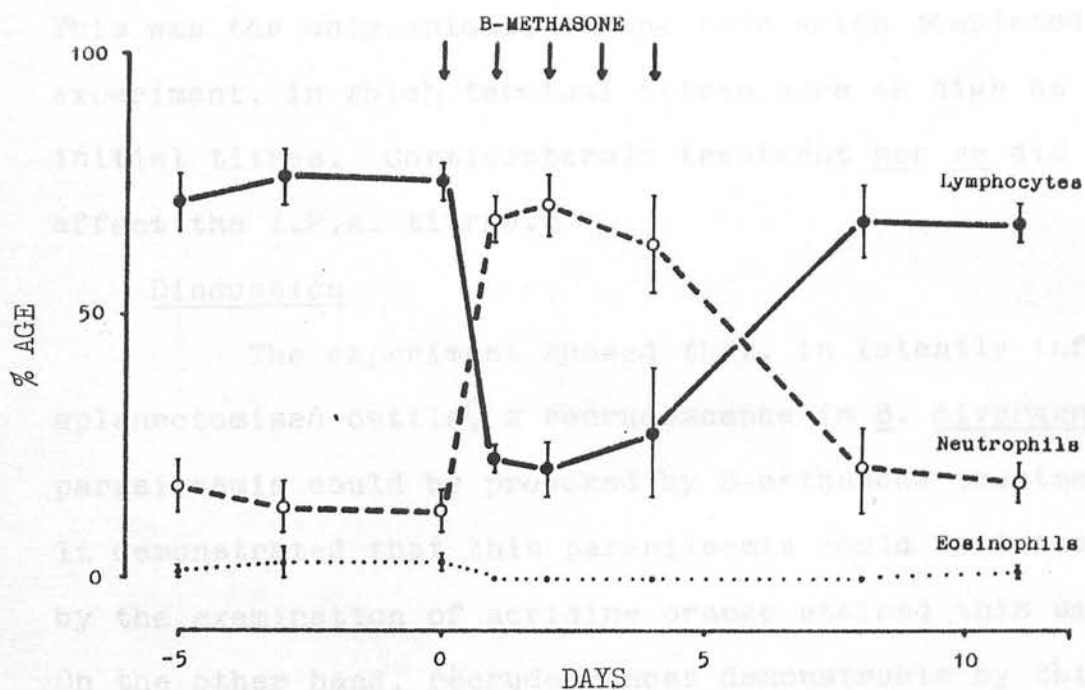


Fig. 16

The effect of B-methasone on percentage differential leucocyte counts in cattle. (Vertical bars show standard deviations of the means from five animals).



to pre-infection levels by day 8.

The non-treated animal did not show any changes in leucocyte picture or blood glucose levels throughout the experiment.

I.F.A. titres during the course of the experiment are plotted in Fig. 17. Titres declined steadily except in one animal (X3) which suffered a relapse parasitaemia. This was the only animal, of the four which completed the experiment, in which terminal titres were as high as initial titres. Corticosteroid treatment per se did not affect the I.F.A. titres.

### Discussion

The experiment showed that, in latently infected splenectomised cattle, a recrudescence in B. divergens parasitaemia could be provoked by B-methasone treatment. It demonstrated that this parasitaemia could be detected by the examination of acridine orange stained thin smears. On the other hand, recrudescences demonstrable by this means could not be provoked in non-splenectomised cattle, even though these had been infected a relatively short time previously. It was therefore decided to use splenectomised cattle for future experiments.

The effects of B-methasone treatment on the leucocyte picture were found to be very marked, in common with previous reports of the effects of corticosteroids (Kerr, Robertson & McGirr, 1951).

Blood glucose levels were significantly elevated by

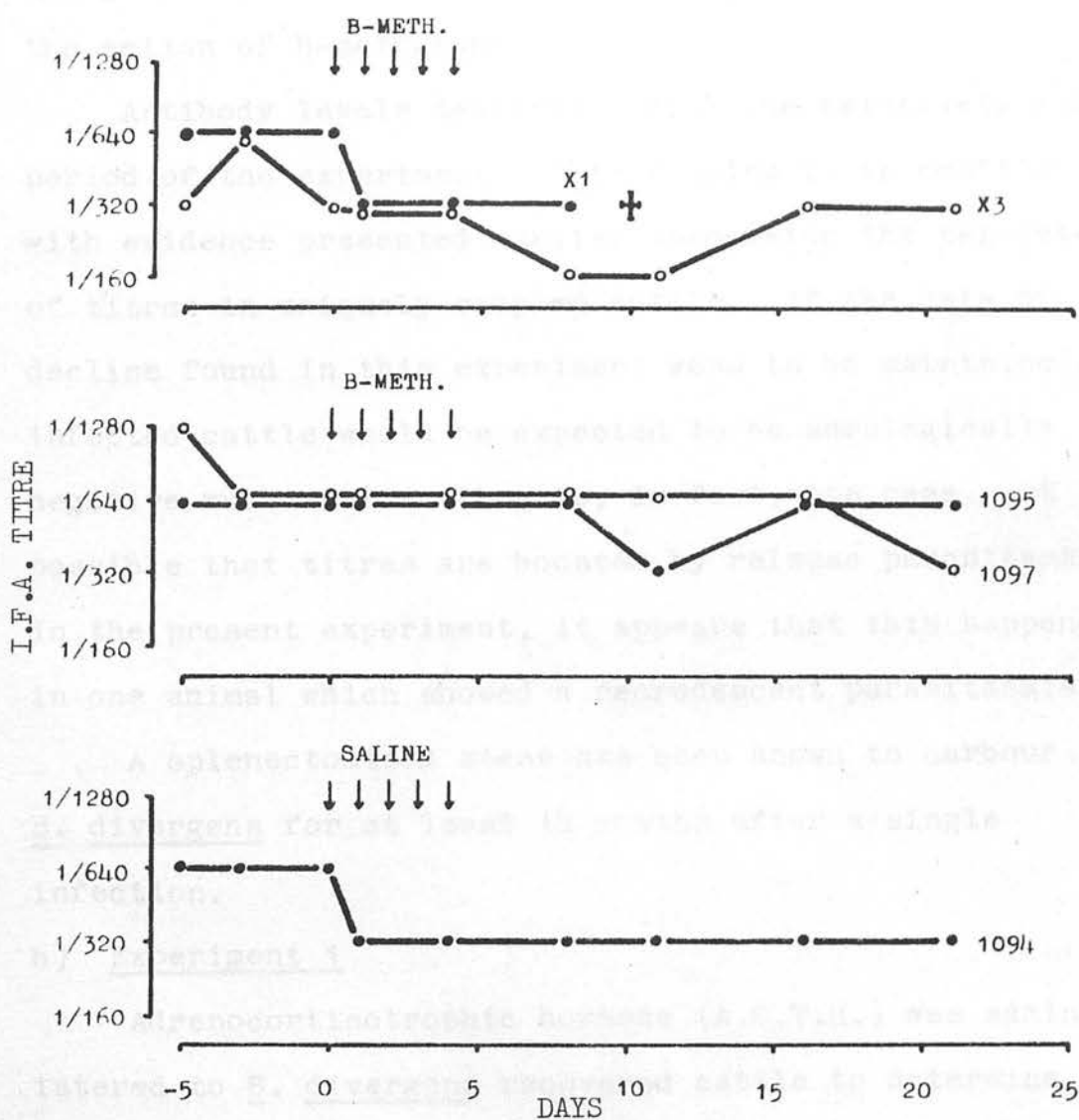


Fig. 17

I.F.A. titres in B. divergens recovered cattle

subjected to B-methasone administration

corticosteroid therapy but it was decided that in future experiments, their assay would be unnecessary, since changes in leucocytes would be sufficient indication of the action of B-methasone.

Antibody levels declined during the relatively short period of the experiment. This finding is in conflict with evidence presented earlier concerning the persistence of titres in uniquely sampled cattle. If the rate of decline found in this experiment were to be maintained, infected cattle would be expected to be serologically negative much earlier than is, in fact, the case. It is possible that titres are boosted by relapse parasitaemias. In the present experiment, it appears that this happened in one animal which showed a recrudescent parasitaemia.

A splenectomised steer has been shown to harbour B. divergens for at least 14 months after a single infection.

b) Experiment 1

Adrenocorticotrophic hormone (A.C.T.H.) was administered to B. divergens recovered cattle to determine the effect on chronic parasitaemias of raising endogenous corticosteroid concentrations.

Materials and methods

Six splenectomised steers, which had recovered from B. divergens infection  $4\frac{1}{2}$  months or 6 months previously, were used. (Five were infected in experiment B.d.2 and one in experiment B.d.1, see Table 16).

Three animals were treated with A.C.T.H. ('Corticotrophin ZN', Organon Laboratories, Morden, Surrey) which was injected intramuscularly, twice daily at 1000 and 1800 hours, for four days, at a dose of 30 i.u./100 kgms. weight each injection.

The first day of treatment was day 0. Blood sampling began on day -1 and continued for 22 days, and the following observations were made daily throughout that period:- parasitaemia, temperature, P.C.V. and W.B.C. counts. Differential W.B.C. and direct eosinophil counts were made daily from day -1 to day 7 (twice on day 0) and less frequently thereafter.

Plasma cortisol assays were performed on days -1 to 5. During the period of injections (days 0-3 inclusive) cattle were also bled at 1200 hours for cortisol estimations.

### Results

The response of parasitaemias is shown in Fig. 18. Recrudescent parasitaemias were observed in all six animals within ten days of the start of the experiment. The initial relapse parasitaemias were higher in the A.C.T.H. treated cattle than in the saline treated animals. In four animals there was evidence of a second relapse. The mean relapse interval was  $11.7 \pm 2.2$  days.

Recrudescent parasitaemias were not accompanied by clinical signs, pyrexia or depression in P.C.V.

Plasma cortisol levels were increased two hours

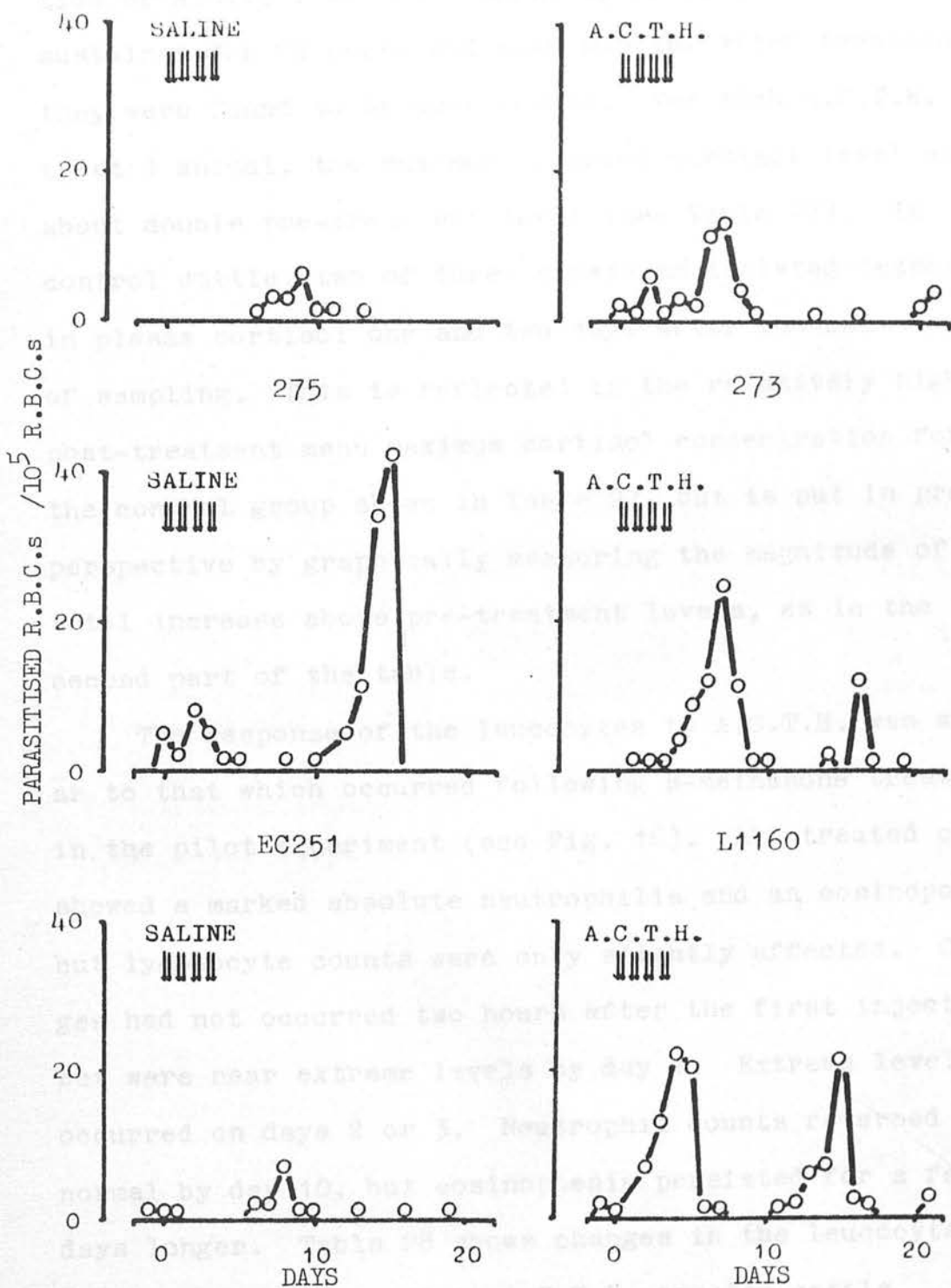


Fig. 18

The effect of A.C.T.H. on B. divergens  
parasitaemias in recovered cattle

after A.C.T.H. injection, but in spite of a second injection of A.C.T.H. at 1800 hours, these levels were not sustained for 24 hours and each morning after treatment, they were found to be near normal. For each A.C.T.H. treated animal, the maximum observed cortisol level was about double pre-treatment level (see Table 27). In control cattle, two of three showed an isolated increase in plasma cortisol one and two days after the commencement of sampling. This is reflected in the relatively high post-treatment mean maximum cortisol concentration for the control group shown in Table 27, but is put in proper perspective by graphically measuring the magnitude of total increase above pre-treatment levels, as in the second part of the table.

The response of the leucocytes to A.C.T.H. was similar to that which occurred following B-methasone treatment in the pilot experiment (see Fig. 16). All treated cattle showed a marked absolute neutrophilia and an eosinopenia, but lymphocyte counts were only slightly affected. Changes had not occurred two hours after the first injection but were near extreme levels by day 1. Extreme levels occurred on days 2 or 3. Neutrophil counts returned to normal by day 10, but eosinopenia persisted for a few days longer. Table 28 shows changes in the leucocyte picture which occurred in A.C.T.H. treated cattle.

The haematological parameters in control cattle did not alter during the experiment except in one animal which



Table 27

Plasma cortisol concentrations in three cattle before and after  
A.C.T.H. treatment (30 i.u./100 kgms. weight/twice daily)

	Plasma cortisol concentration ( $\mu$ gms./100 mls.)		Magnitude of total increase in plasma cortisol*	
	Pre-treatment		Post-treatment†	
A.C.T.H.	5.2 $\pm$ 1.9	Sig. 5%	10.0 $\pm$ 2.3	9.6 $\pm$ 3.4 Sig. 5%
CONTROL	4.7 $\pm$ 0.8	N.S. 5%	7.5 $\pm$ 1.8	1.3 $\pm$ 0.7

\* - Measured graphically as the area enclosed by the response curve  
above pre-treatment level and expressed in arbitrary units

† - Mean of post-treatment maxima

N.S. - Not significant

Table 28

Total leucocyte and absolute differential counts in three cattle before and after A.C.T.H. treatment (30 i.u./100 kgms. weight/twice daily) (counts  $\times 10^3/\text{cu. mm.}$ )

	Total leucocytes	Lymphocytes	Neutrophils	Eosinophils
Pre-treatment	$13.1 \pm 2.6$	$10.5 \pm 2.0$	$3.4 \pm 1.0$	$0.25 \pm 0.07$
	Sig. 5%	N.S. 5%	Sig. 1%	Sig. 1%
Post-treatment extremes	$28.7 \pm 6.1$	$8.1 \pm 1.9$	$16.9 \pm 3.9$	$0.008 \pm 0.006$

had shown a temporary rise in plasma cortisol concentration. This was followed by a transitory eosinopenia. Leucocytic changes did not occur concurrent with recrudescient parasitaemias not associated with A.C.T.H. treatment.

Antibody titres, measured at strategic intervals, declined during the experiment (Fig. 19). There is no consistent difference in the pattern of decline between A.C.T.H. or control groups, nor between those animals which suffered relatively large and multiple recrudescient parasitaemias and those which showed small single recrudescences.

### Discussion

The experiment demonstrated that in splenectomised calves infected with B. divergens, patent recrudescences in parasitaemia can occur in control animals given injections of normal saline. Compared with control injections, treatment with A.C.T.H. produced marked haematological changes and increased plasma cortisol levels. Recrudescient parasitaemias were of greater magnitude in A.C.T.H. treated animals. However, second relapse parasitaemias were seen, in both A.C.T.H. and control groups, apparently unrelated to any foregoing treatment. The greatest relapse which was observed occurred in a control animal. The observed relapse interval was fairly uniform, suggesting that spontaneous relapses may occur with a regular periodicity.

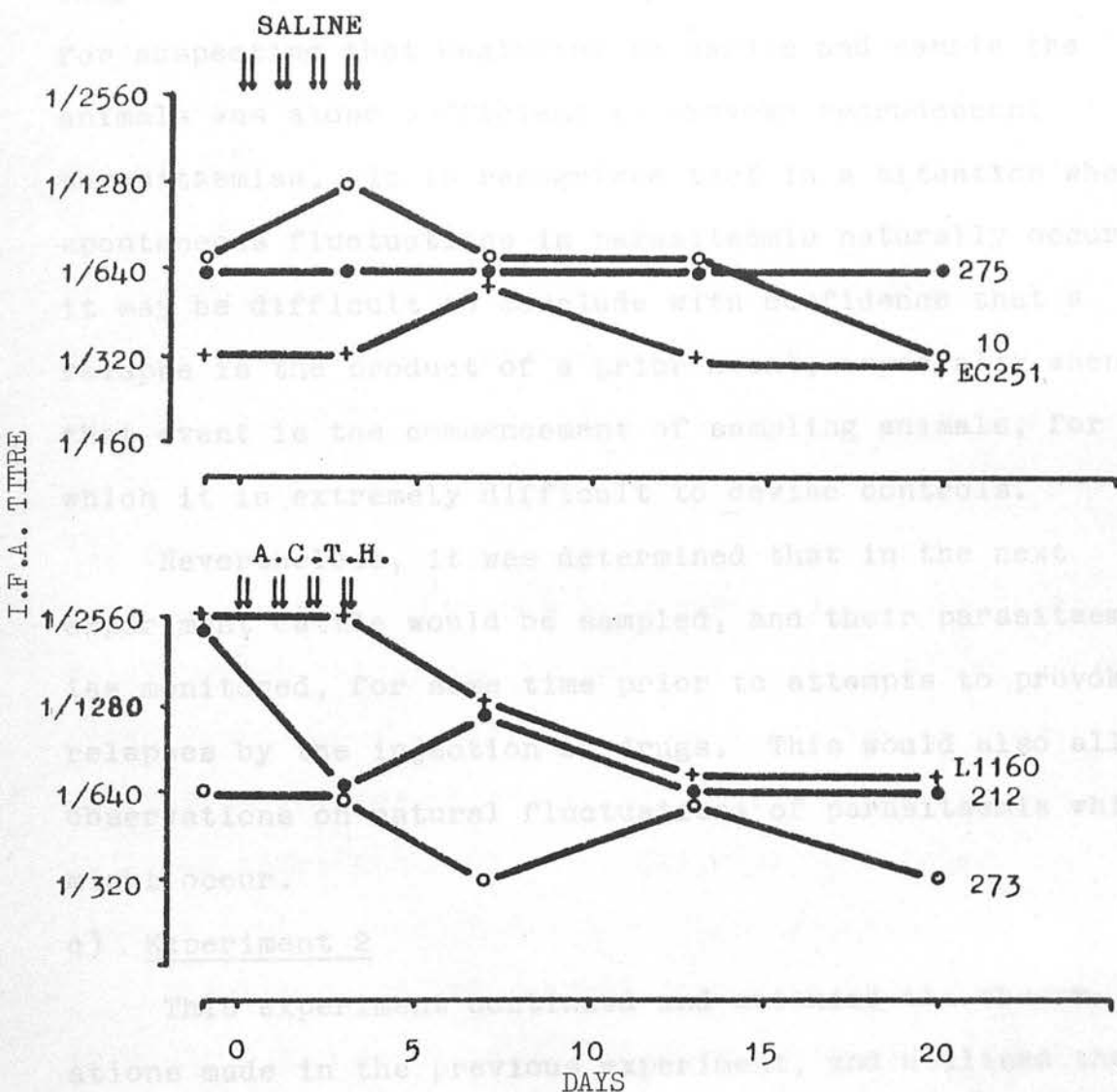


Fig. 19

I.F.A. titres in B. divergens recovered cattle  
subjected to A.C.T.H. and control injections

Whilst the commencement of handling and sampling was not associated with marked haematological changes suggestive of stress, and although plasma cortisol was elevated only briefly in two animals, this experiment gives grounds for suspecting that beginning to handle and sample the animals was alone sufficient to provoke recrudescent parasitaemias. It is recognised that in a situation where spontaneous fluctuations in parasitaemia naturally occur, it may be difficult to conclude with confidence that a relapse is the product of a prior event, especially when that event is the commencement of sampling animals, for which it is extremely difficult to devise controls.

Nevertheless, it was determined that in the next experiment cattle would be sampled, and their parasitaemias monitored, for some time prior to attempts to provoke relapses by the injection of drugs. This would also allow observations on natural fluctuations of parasitaemia which might occur.

c) Experiment 2

This experiment continued and extended the observations made in the previous experiment, and utilised the same six cattle after a rest period. Parasitaemias in the cattle were monitored for some time before A.C.T.H. was injected. The dose of A.C.T.H. was increased in an attempt to provoke sustained elevated plasma cortisol concentrations and later, B-methasone was administered to assess its effect in the light of the failure of

A.C.T.H. to provoke relapse parasitaemias.

### Materials and methods

The same six cattle as were used in the previous experiment were observed for a continuous period of 75 days. Sampling was begun on day 0. The previous experiment had terminated 24 days earlier and the cattle had not been handled between that time and day 0.

On day 23, three cattle (those which had been used as controls previously) were given A.C.T.H. injections and the other three received similar injections of normal saline. A.C.T.H. was administered intramuscularly, once daily at 10<sup>00</sup> hours, for four days at a dose of 100 i.u./100 kgms. weight.

On day 57, the groups were reversed and the three cattle which had served as controls on days 23-26, were given B-methasone whilst the other three cattle received control injections of normal saline. B-methasone was injected intramuscularly once daily at 10<sup>00</sup> hours, for four days at a dose of 0.1 mgms./kgm. weight.

Parasitaemias and temperatures were determined daily throughout the experiment. Haematological parameters, plasma cortisol levels and antibody titre were determined at selected times before, during and after the administration of drugs.

### Results

Cattle tolerated, without illness, the dosages of A.C.T.H. and B-methasone administered.



The parasitaemias observed throughout the experiment are shown individually in Fig. 20. Throughout the observation period, parasitaemias fluctuated with a striking degree of regular periodicity. The administration of A.C.T.H. and B-methasone had no apparent effect on the pattern of spontaneous recrudescences. The relapse intervals were very regular except in one animal, EC251. In Table 29, mean relapse intervals are shown. Comparison of mean relapse intervals reveals that statistically significant differences occurred between different animals. Table 30 combines the results of two previous experiments and shows the relapse intervals observed for each animal at previous stages in its infection. The relapse interval in four cattle did not change between two separate experiments at  $4\frac{1}{2}$  and 6 months post-infection. The first relapse interval, immediately following maximum primary parasitaemia, is however shorter for each animal than later relapse intervals. Nevertheless, calves which had the longest relapse intervals late in infection also had the longest first relapse intervals.

In the present experiment, four of the six cattle suffered recrudescence parasitaemias immediately after sampling.

The relapse parasitaemias were not accompanied by clinical signs of disease, pyrexia or any depression in P.C.V.

Mean plasma cortisol concentrations for the A.C.T.H.

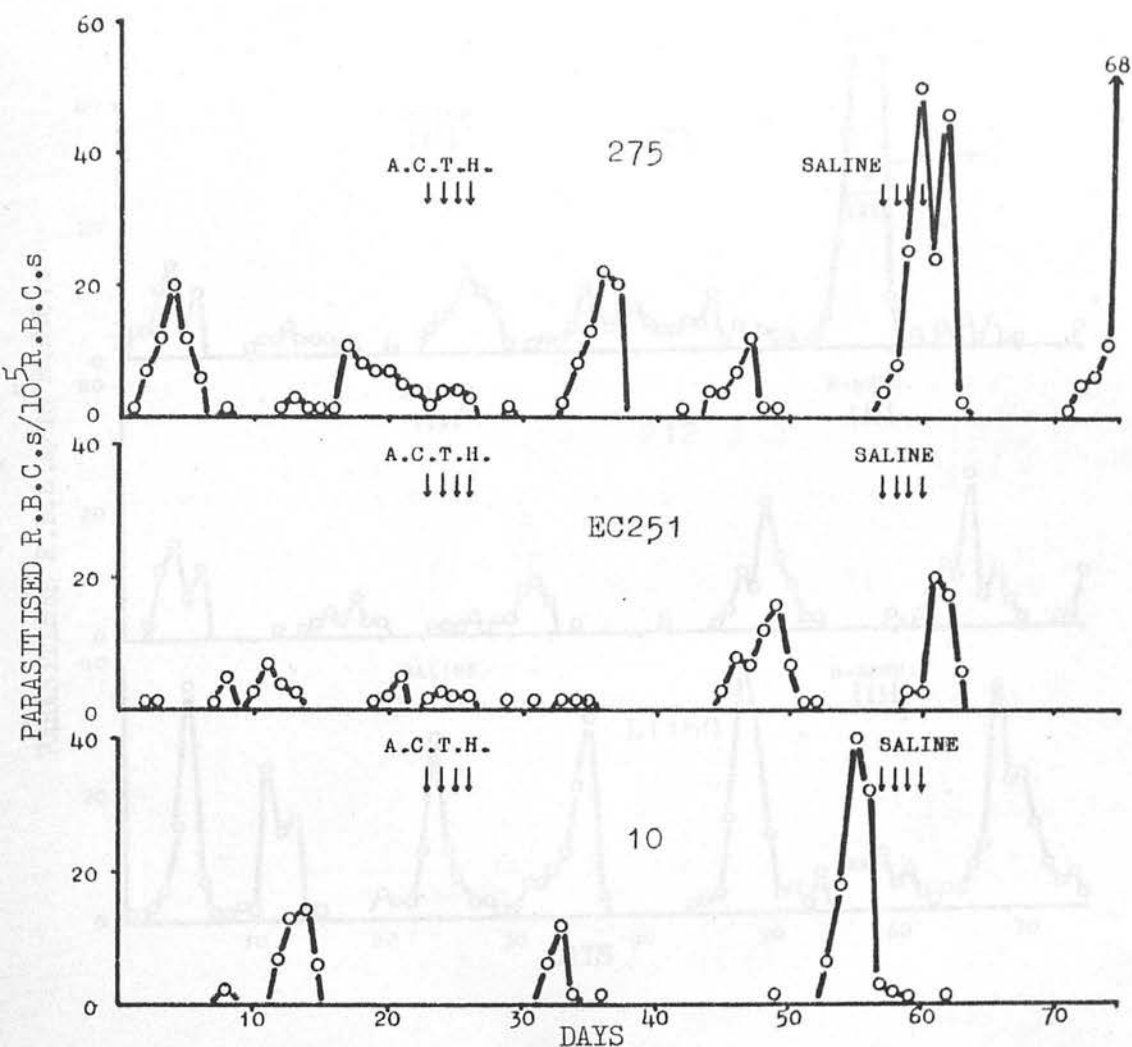


Fig. 20a

B. divergens parasitaemias in recovered cattle and the effect of A.C.T.H. administration upon them.

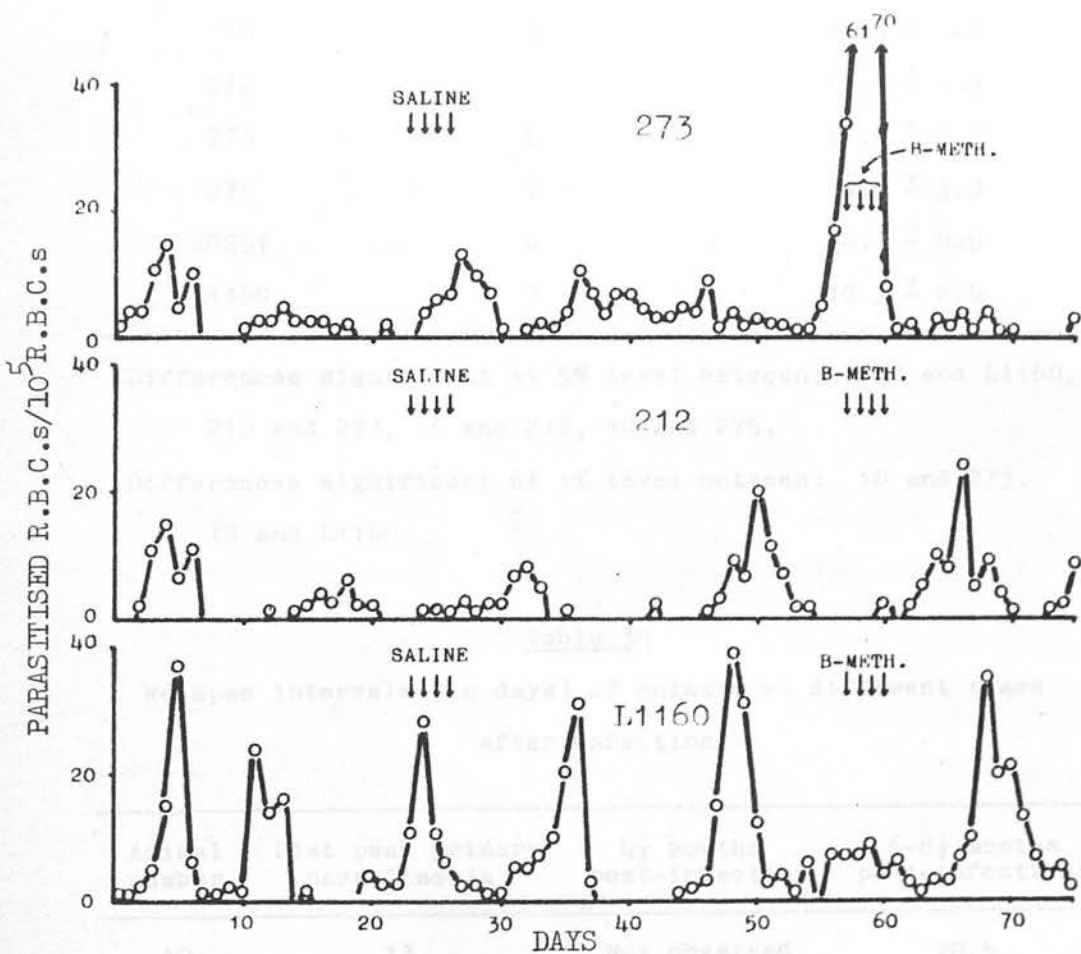


Fig. 20b

B. divergens parasitaemias in recovered cattle and the effect of B-methasone administration upon them.

Table 29

Relapse intervals of individuals in a continuously observed group of cattle

Animal number	Number of relapses	Mean relapse interval (days)
10	3	20.5 $\pm$ 2.1
212	5	15.5 $\pm$ 1.9
273	6	11.0 $\pm$ 2.3
275	6	14.2 $\pm$ 3.0
EC251	4	16.7 $\pm$ 9.9
L1160	7	10.5 $\pm$ 2.6

Differences significant at 5% level between: 212 and L1160, 212 and 273, 10 and 212, 10 and 275.

Differences significant at 1% level between: 10 and 273, 10 and L1160.

Table 30

Relapse intervals (in days) of animals at different times after infection

Animal number	Post peak primary parasitaemia	4½ months post-infection	6-8½ months post-infection*
10	13	Not observed	20.5
212	8	14+	15.5
273	Not observed	9	11.0
275	8	13	14.2
EC251	12	Not observed	16.6
L1160	Not observed	11	10.5

\* - Mean values

treated and control groups are shown in Fig. 21. A.C.T.H. raised cortisol concentrations within two hours of administration, and the elevated levels were reasonably well sustained for 24 hours, with the result that a constant plasma cortisol concentration, two to three times in excess of initial levels, was maintained for five days. The control cattle also showed raised plasma cortisol concentrations during the period of injections. That this was a real increase rather than a drift in experimental error was suggested by the fact that assays on 'Seronorm' standards remained within uniform and predicted values.

In spite of the increase in plasma cortisol concentrations, the white blood cell picture in the controls was not affected. In A.C.T.H. treated animals, the by now familiar changes occurred. There was a profound leucocytosis, a crossover in the lymphocyte/neutrophil ratio, a neutrophilia, an eosinopenia and a lymphocytopenia.

The extent of the changes is shown in Table 31.

During the period of B-methasone administration, plasma cortisol levels declined slightly in both control and treated animals. The extent of the change was similar in both groups, suggesting a trend in technical error, but again, 'Seronorm' standards remained within acceptable limits. Compared with control animals, there was no indication in B-methasone treated cattle that endogenous cortisol concentrations were depressed.

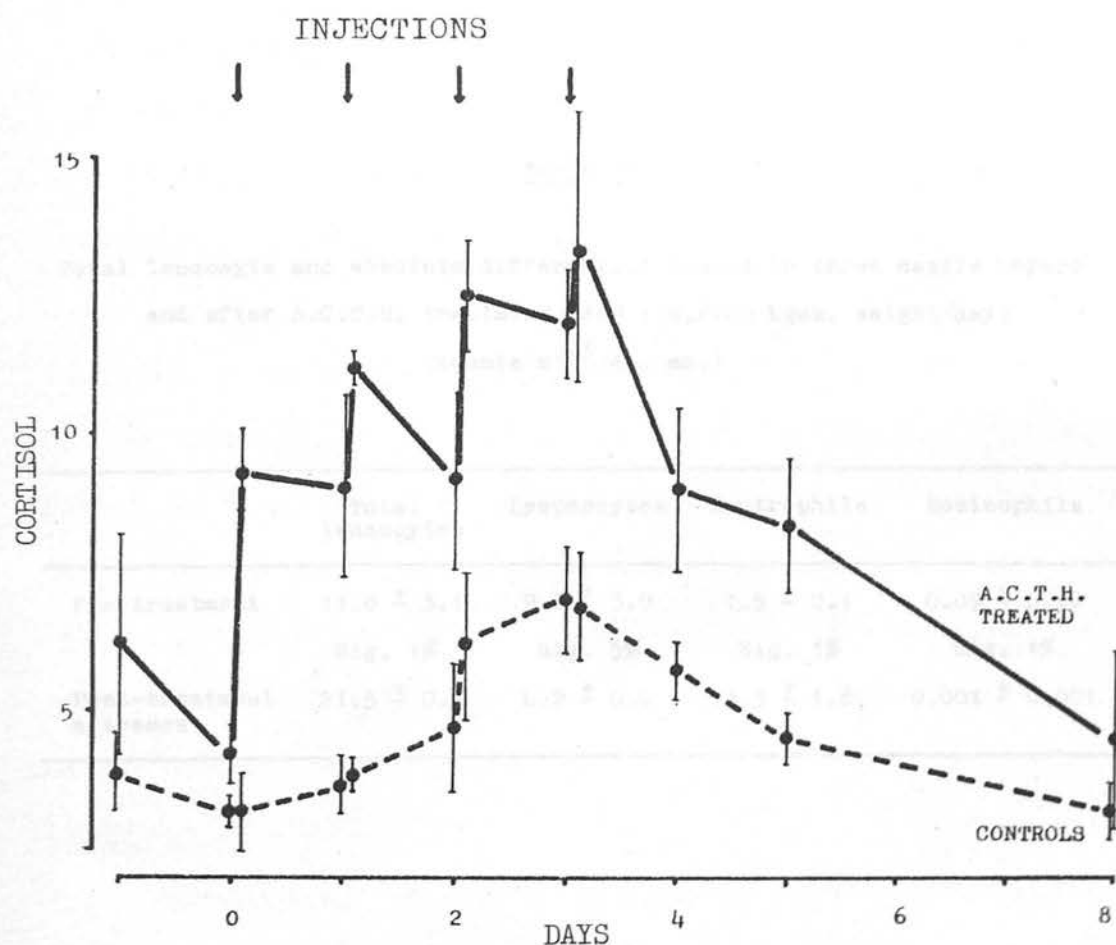


Fig. 21

The effect of A.C.T.H. administration (100 i.u./100 kgms. weight/day) on plasma cortisol concentrations ( $\mu$ gms./100 mls.) in cattle. Group means and standard deviations (vertical bars) shown - three animals per group.



Table 31

Total leucocyte and absolute differential counts in three cattle before and after A.C.T.H. treatment (100 i.u./100 kgms. weight/day) (counts  $\times 10^3/\text{cu. mm.}$ )

	Total leucocytes	Lymphocytes	Neutrophils	Eosinophils
Pre-treatment	$11.8 \pm 3.1$	$9.6 \pm 3.0$	$1.5 \pm 0.1$	$0.09 \pm 0.02$
	Sig. 1%	Sig. 5%	Sig. 1%	Sig. 1%
Post-treatment extremes	$21.5 \pm 0.4$	$4.2 \pm 0.4$	$13.3 \pm 1.8$	$0.001 \pm 0.001$

## Discussion

Few studies have been conducted on the parasitocidal in cattle which have recovered from *S. divergens* infection. Joyner & Davies (1967) showed that infection was retained for up to 4 years in non-splenectomized calves and for up to 5 years in splenectomized calves. After the elimination of parasites as judged by parasitological calves were still immune to challenge.

The present work indicates that splenectomized calves will retain parasitocidal for up to 500 days at levels detectable by blood smear examination, and that chronic

B-methasone treatment caused profound changes in the leucocyte picture which are summarised in Table 32. There was a leucocytosis, a crossover in the lymphocyte/neutrophil ratio, a neutrophilia and an eosinopenia. A slight lymphocytopenia occurred but it was not statistically significant.

When first determined on day 8 for the six cattle, antibody titres varied between 1/160 and 1/890 (geometric mean 1/375). By day 70 they were no different, having the same group geometric mean titre and the same range. During the course of the experiment, individual titres fluctuated within a range sometimes extending to two dilution intervals i.e. fourfold. However, fluctuations were irregular and were not related to recrudescent parasitaemias, and neither treatment with A.C.T.H. nor B-methasone had any apparent effect on antibody levels.

### Discussion

Few studies have been conducted on the parasitaemias in cattle which have recovered from B. divergens infection. Joyner & Davies (1967) showed that infection can be retained for up to 4 years in non-splenectomised calves and for up to 6½ years in splenectomised calves. After the elimination of parasites, as judged by subinoculation, calves were still immune to challenge.

The present work indicates that splenectomised calves will retain parasitaemias for up to 500 days at levels detectable by blood smear examination, and that chronic

Table 32

Total leucocyte and absolute differential counts in three cattle before and after B-methasone treatment  
(counts  $\times 10^3/\text{cu. mm.}$ )

	Total leucocytes	Lymphocytes	Neutrophils	Eosinophils
Pre-treatment	$13.4 \pm 4.8$	$9.7 \pm 3.1$	$2.7 \pm 1.5$	$0.2 \pm 0.06$
	Sig. 5%	N.S. 5%	Sig. 1%	Sig. 1%
Post-treatment extremes	$32.1 \pm 5.3$	$4.8 \pm 2.2$	$19.2 \pm 5.0$	$0.004 \pm 0.001$

parasitaemias fluctuate at regular and frequent intervals. Barnett (1965) remarks that parasitaemias of B. trautmani, presumably immediately after first infection, showed a series of peaks 6 to 8 days apart before ultimately becoming sub-patent. Relapse parasitaemias immediately after splenectomy have frequently been observed in various Babesia spp. infections. In cattle infected with B. bigemina, Sargent, Donatien, Parrot & Lestoquard (1945) observed a series of recrudescent parasitaemias following splenectomy. In splenectomised rats infected with B. rodhaini, Phillips (1969b) observed secondary and tertiary parasitaemias. Irregular and relatively infrequent relapse parasitaemias occur in B. argentina infected non-splenectomised cattle not exposed to re-infection (Mahoney, 1972 and Curnow, 1973b). Data presented by Ewing (1965) of his prolonged observations of B. canis parasitaemias in dogs, show a cyclic pattern of parasite recrudescences. Barnett (1965) suggested that regular relapses probably involved antigenic variants, and work by Brown & Brown (1965) and Gray (1965) has shown this to be the case in Plasmodium knowlesi and Trypanosoma brucei infections. Using a strain specific agglutination test, Curnow (1973b) has recently demonstrated that naturally occurring relapse parasitaemias of B. argentina in a calf were antigenically different. From this evidence, it seems highly likely that the relapses noted in B. divergens infected calves were of antigenically variant

populations. Proof of this would not be easy to obtain, because of the difficulty in preparing antigen from relapse parasitaemias. Curnow (1968) was able to concentrate low parasitaemias by the method of selective lysis using hypotonic saline, but, in the present study, this has been shown to be inapplicable to B. divergens. Gray (1965) made isolates of T. brucei into laboratory hosts but this would not be feasible with B. divergens. Relapse populations would therefore have to be isolated in separate splenectomised calves.

In view of the fact that relapse parasitaemias were observed on at least two separate occasions in individual splenectomised calves (see Table 30), it seems likely that the parasitaemias were continuously fluctuating. Because chronic parasitaemias are maintained at much lower levels in non-splenectomised cattle, it has not been possible to observe how these parasitaemias behave. The fact that such cattle suffered a patent recrudescent parasitaemia immediately after primary parasitaemia suggests that cyclic parasitaemias also occur in non-splenectomised cattle infected with B. divergens.

How might cyclic parasitaemias be produced? If variants of the parent population were being randomly produced and were multiplying, the effect of a large number of superimposed asynchronous parasitaemias would be observed as a continuous, relatively stable parasitaemia. This is patently not the case. As Ross & Mahoney

(1974) have suggested, the occurrence of defined recrudescences of parasitaemia implies that variants arise one at a time in sequence (or possibly several variants arise synchronously on each occasion). There appear to be two alternative mechanisms which could produce this pattern. The essence of the first alternative is that the full variant potential of the parasite is continuously present as an antigenic pool from which variants selectively multiply. This theory implies a complex system of suppression of all but one (or a few) of the variants at any one time. It is known that different species of haemo-parasite will suppress rising parasitaemias of Babesia spp. (Hoyte, 1961; Peters, 1965 and others) and it is conceivable that different strains within the same species might exhibit a similar degree of interference. Herbert (1975) has produced evidence that this does occur with strains of Trypanosoma brucei. This selective theory fits rather well with the idea of immunosuppression provoking relapse parasitaemias.

The second alternative is that antigenic variation is actively provoked in a sequential manner notably by the action of antibody. Phillips (1969a), Thoongsuwan & Cox (1973) and Roberts & Tracey-Patte (1975b) have all shown that immune serum will affect B. rodhaini in this way. Roberts & Tracey-Patte (1975b), using clones of parasites, found that a stable heritable change was induced by immune sera and that the effect was related to



the specificity and concentration of the antibody. They used the term 'immuno-induction' to describe this phenomenon. If the induction of antigenic variation is responsible for parasitaemic recrudescences, this implies that extrinsic factors which provoke recrudescences do so by a positive effect on the parasite. The paradoxical corollary of this is that, unless they have a direct action on the parasite, immunosuppressants may actually reduce the pressure of variant induction and hence the frequency of recrudescences.

Whichever alternative is operative, the minimum relapse interval will be determined by the speed of the protective antibody response of the host. By a selection theory, this response would eliminate the variant population and remove the source of suppression on the antigenic pool, and by the theory of 'immuno-induction' new variants would not be induced until antibody against the existing population was present.

Susceptible animals exposed to primary infection are analogous to premune animals faced with a new variant strain. Our observations on the dynamics of the antibody response to B. divergens show that splenectomised calves begin to produce IgG antibody 12-14 days after infection. This time period agrees remarkably well with the observed relapse interval (see Tables 29 and 30). The antibodies measured by the I.F.A. test are not necessarily protective, but in our experiments the appearance of I.F.

antibody coincided with maximum parasitaemia. The latter presumably coincides with the appearance of protective antibody. From this, one would predict that the minimum relapse interval for a given Babesia spp. in a given host would be similar to the time to maximum primary parasitaemia, and that the relapse intervals of different Babesia spp. would bear the same relationship to one another as would their times to peak parasitaemia (assuming that the hosts and the numbers of parasites inoculated were comparable). For instance, the relapse interval for B. bigemina would be short compared with that for B. divergens. It would be interesting to examine other haemoparasites characterised by persistent parasitaemias. By analogy and if the same mechanisms as have been conjectured are operative, the minimum relapse interval for Anaplasma marginale should be very long (e.g. 20 days or more). This phenomenon may be fully manifest only in splenectomised hosts. An examination of Ewing's (1965 and 1966) data pertaining to chronic B. canis infections in splenectomised dogs, shows a relapse interval of 12-16 days. Ewing monitored parasitaemias daily and this would appear to be the minimum relapse interval for B. canis.

The occurrence of a recrudescent peak, is circumstantial evidence that a variant, which is alien to the host, has been produced. By inference, the number of recrudescences indicates the potential for antigenic variation of the parasite (Ross & Mahoney, 1974). It has

been reasoned that parasitaemias may have relapsed continuously, even when not observed, in the B. divergens infected calves. If this is true, then the number of antigenic variants produced following a single infection was as many as 25. At the end of the observation period, there was no sign that the potential for variation was being exhausted.

It is not possible to know definitely whether the parasitaemias continued to relapse between observation periods. At that time, the behaviour of the parasitaemia may have been different, because there is some evidence from our observations that it was modified by the conduct of the experiments. In the final experiment, 4/6 calves relapsed from a state of latency to a peak of recrudescence 4-5 days after observations were commenced. The probability that this would happen in one calf, with a relapse interval of say 12 days, is about 0.2. That it should happen in 4/6 calves in a group is therefore most unlikely by chance alone (about 0.002), unless the parasitaemias were cycling synchronously. Parasitaemias are unlikely to be cycling synchronously six months after infection, since individual variations in relapse interval (which can be significant - see Table 29) will have long since obliterated the effect of simultaneous infection. The observation therefore implies that the commencement of handling animals can provoke recrudescent parasitaemias. This is not altogether surprising, since the hand-

ling of cattle has been shown to exert subtle effects in other ways. For instance, red cell parameters are altered, even though leucocytic changes indicative of a stress response are limited (Gartner, Callow, Granzien & Pepper, 1969). In control animals of Experiment 1, such leucocytic changes were also minimal, though a transient increase in plasma cortisol occurred in two calves. One of these calves relapsed at 3 days and the other two at 9 and 10 days (peak times) after the commencement of the experiment. The latter two animals (10 and EC251) were later shown to be 'slow' relapsers (Table 30), so it seems feasible that these recrudescences were also a result of the commencement of observations.

Another observation, which may reflect an influence of sampling the cattle, was that recrudescence parasitaemias tended to increase in magnitude during the 75 day observation period. This is inconsistent with the general rule of parasitaemia, in infrequently sampled animals, falling gradually to extinction.

Injections of A.C.T.H. produced profound changes in the leucocyte picture and elevated plasma cortisol levels significantly. In Experiment 1, this appeared to exacerbate the first relapse parasitaemias. In Experiment 2, when administered 23 days after sampling was begun, it appeared to have no effect on the chronic parasitaemias. Similarly in Experiment 2 B-methasone caused extreme leucocytic changes, but had no effect on the pattern of

relapsing parasitaemias. In one case, when administration coincided with a rising relapse parasitaemia, the peak of the relapse was greater than was expected. This effect is analogous to the influence of corticosteroid administered during primary infection (Leeflang & Perié, 1972).

The failure of A.C.T.H. to provoke relapses is not surprising in the light of the fact that B-methasone proved equally ineffective. Why this should be so, is puzzling, and is in contradiction with the results of our own pilot experiment, and those of Callow & Parker (1969) and Hoffmann, Schein & Müller (1971) who were working with B. argentina and B. bigemina respectively. In all of these experiments, corticosteroid was administered at the beginning of the observation period. Furthermore, Callow & Parker (1969) performed brain biopsies immediately before treatment. This involved the restraint of cattle in a special 'bail' and the drilling of the cranium. In these experiments, it is therefore possible that the administered B-methasone merely exacerbated recrudescences which the stress of sampling had provoked. This effect was seen in our own Experiment 1, where all six cattle relapsed after sampling, but recrudescences were greatest in those calves coincidentally given A.C.T.H. Callow & Parker (1969) were using non-splenectomised cattle and they did not observe relapses in control animals. This could be explained if the threshold for detection fell above those parasitaemias provoked in



control animals but below the exacerbated parasitaemias of B-methasone treated animals.

Undoubtedly the effect of corticosteroids on chronic Babesia spp. infections is more complex than may have been imagined. It is clear that experiments involving a study of their effect (and the effect of any other factors too), must include a preliminary period of observation of at least two weeks in order to prevent the effect of sampling the host from influencing the response of the parasite.

Neither B-methasone nor A.C.T.H. had a detectable effect on antibody levels. Work with various antigen-antibody systems has shown that corticosteroids are potent inhibitors of the humoral antibody response in certain species e.g. mouse, rat and rabbit - although relatively little data are available with respect to other species (Bach, 1975). Most studies have been concerned with the effect of corticosteroids on the primary antibody response rather than on pre-existing antibody levels in the blood, but it has been shown that the rate of antibody protein destruction is not affected by cortisone (see McMaster & Franzl, 1961). Working with Theileria sergenti in cattle, Takahashi, Yamashita & Shimizu (1975) did not detect any change in I.F.A. titre after corticosteroid administration to chronically infected animals, although recrudescent parasitaemias were provoked. It is appropriate to note at this point that Sibinovic, Sibinovic, Ristic & Cox



(1967) have shown that spontaneous recrudescent Babesia spp. parasitaemias are closely associated with depressions in antibody titre to serum antigens.

Recrudescent parasitaemias were not followed by detectable changes in antibody titre. This is not, perhaps, surprising in view of the persistence of  $\gamma$  - globulins (half-life 5-6 days, A.G. Luckins, pers. comm.) and the lack of strain specificity of the I.F.A. test. Persisting antibodies would mask any rise in strain specific antibody which might occur. Frequent relapses would, however, be expected to maintain I.F.A. titres, compared with a non-relapsing situation.

be gained by examining the few relevant published papers and the Annual Reports of the Veterinary Department. This picture is necessarily limited and superficial by virtue of the nature of the disease reports, which in most cases are very brief, and of course reflect only those cases presented to the veterinary department. The dearth of research into babesiosis is understandable, in view of its relatively minor significance compared with the major epizootic diseases such as rinderpest and contagious pleuropneumonia, which were formerly of much importance in Nigeria. However, these diseases are now either eradicated or very limited in occurrence, and babesiosis has become a relevant and appropriate subject for research in Nigeria, especially in view of the increasing interest in the importation of exotic breeds of

C. Babesia bigemina

Introductory review of bovine babesiosis in Nigeria

Babesia bigemina is a large parasite first described and incriminated as the cause of 'Texas fever' by Smith & Kilborne (1893) in the southern states of North America. Since then it has been discovered as a parasite and pathogen of cattle throughout the tropics. Studies on babesiosis in Africa south of the Sahara have largely been concentrated in East and South Africa and little fundamental research appears to have been conducted in West Africa. An impression of the occurrence and distribution of Babesia spp. parasites and babesiosis in Nigeria can be gained by examining the few relevant published papers and the Annual Reports of the Veterinary Department. This picture is necessarily limited and superficial by virtue of the nature of the disease reports, which in most cases are very brief, and of course reflect only those cases presented to the veterinary department. The dearth of research into babesiosis is understandable, in view of its relatively minor significance compared with the major epizootic diseases such as rinderpest and contagious pleuropneumonia, which were formerly of such importance in Nigeria. However, these diseases are now either eradicated or very limited in occurrence, and babesiosis has become a relevant and appropriate subject for research in Nigeria, especially in view of the increasing interest in the importation of exotic breeds of

cattle which may be susceptible to the disease.

Bovine piroplasmosis has been diagnosed in Nigeria from the earliest days of the veterinary services, and since then has been recognised as an endemic infection in native cattle which, in normal circumstances, causes relatively little disease. On occasions babesiosis - especially in conjunction with Theileria mutans or trypanosomiasis - has been suspected as a cause of unthriftiness in adult stock (Nigeria, 1930) and of mortality in native calves (Nigeria, 1950-51).

Babesia bigemina has been identified in bovine blood smears regularly since at least 1914 (MacFie, 1914). Clinical babesiosis has occurred sporadically throughout the years. In view of the successful use of trypan blue for treatment, it appears that B. bigemina has been responsible for these outbreaks, although this is rarely stated in the reports.

B. bovis has seldom been observed during routine diagnostic examination of blood smears, but a recent survey revealed that this parasite is very common. Of 313 cattle killed at Zaria slaughter slab, over 11% were found, by examination of brain crush smears, to be infected with B. bovis (Folkers, Kuil & Perié, 1967).

By far the greatest attention that 'redwater' has received has been in connection with its occurrence after rinderpest vaccination. Rinderpest was a widespread, common and serious disease of cattle in Nigeria and its

control has been the major priority of the veterinary services since their inauguration. Initial control measures using a double-inoculation of rinderpest infected bovine blood and antiserum were accompanied by mortality rates of around 2% which in some cases were much higher (Nigeria, 1934). A large proportion of these deaths, as high as 40% in one instance (Nigeria, 1928a), was associated with 'redwater'. Whilst it was recognised that some of the 'redwater' was a result of the relapse of premune animals or of the natural infection of cattle during visits to immunisation camps, it was demonstrated in the early 1930s that most cases of 'redwater' after vaccination were due to infection of the donor blood with B. bigemina (Nigeria, 1934). In vitro treatment of the blood vaccine with trypan blue greatly reduced the post vaccination mortality rates. Study of the quoted figures (Nigeria, 1934) gives some idea of the susceptibility of the native cattle population. In Sokoto province, of 3,500 cattle vaccinated with untreated blood, 2% died of 'redwater'. The strength of the Babesia spp. challenge cannot of course be ascertained, but it is apparent that even when under the stress of simultaneous vaccination with virulent rinderpest virus, the susceptibility of native cattle was very low. Calves proved more susceptible than older stock since 'redwater' rarely occurred in animals over 2 years old (Nigeria, 1927 and 1928b). In Sokoto province, when 1,500 cattle were vaccinated

with trypan blue treated blood, none died. Similarly in Plateau province, deaths in calves due to 'redwater' following rinderpest vaccination fell from 9.5% to 1.5% after treatment of the blood vaccine (Nigeria, 1934). The incidence of true relapses was therefore low.

After the introduction of dried goat vaccine in the early 1940s, 'redwater' as a sequel to vaccination is not recorded as being a problem in Nigeria, though Branagan (1965 and pers. comm.) describes a vaccine of this type causing true relapses of pathogenic Protozoa, including Babesia spp., during anti-rinderpest campaigns in East Africa.

Bovine babesiosis has been reported from the North Eastern, North Western, Kano, North Central, Kwara, Benue-Plateau and Lagos states. The absence of reports from most of the southern states is more a reflection of the distribution of cattle and veterinary personnel than of the disease. In more recent years, outbreaks involving hundreds of cattle have occurred in Katsina and the disease has been reported as common in the Gombe district near Bauchi (Nigeria, 1963-64).

A seasonal occurrence of ticks and tick-borne diseases during the rains has been reported (Nigeria, 1947).

From this brief review of the available literature relating to babesiosis in Nigeria, it is apparent that the condition is enzootic in cattle in virtually all parts

of the country. Furthermore, the presumed vector in Nigeria, Boophilus decoloratus, is widespread and common (Unsworth, 1952). This epidemiological situation, so characteristic of babesiosis, means that overt disease is rare in native cattle, but that imported cattle are exposed to challenge unless protected from ticks.

The isolation of a Nigerian strain of Babesia bigemina

### Introduction

Babesia spp. parasites exist in one of two hosts - an invertebrate host, the tick, and a vertebrate host. There are therefore two sources from which the parasite can be isolated.

Due to its relatively benign nature and its persistence in the vertebrate host, B. bigemina in all probability chronically infects a majority of unsprayed African cattle which live in areas where vector ticks are prevalent. Serological surveys support this contention (Mehlitz & Ehret, 1974). The subinoculation of blood from indigenous cattle into a susceptible beast therefore affords one possible means of isolating the parasite. The collection of engorged ticks from such cattle and the feeding of their progeny on susceptible cattle provides a second.

In an attempt to isolate a Nigerian strain of B. bigemina, both methods were used.

The most important vectors of B. bigemina are ticks of the genus Boophilus. Neitz (1956) has comprehensively



reviewed the literature relating to the transmission of Babesia spp. In Australia, and Central and South America, Boophilus microplus transmits B. bigemina, whilst in the southern states of North America, Boophilus annulatus was historically the vector of 'Texas fever' (Smith & Kilborne, 1893). In Africa B. bigemina has been shown to be transmitted by both Boophilus decoloratus and Boophilus annulatus, though none of these observations pertains to West Africa.

In Nigeria, the vector of babesiosis is not specifically known, but is presumed to be a boophilid. Three Boophilus species occur in Nigeria. Boophilus decoloratus is widespread and, apart from Amblyomma variegatum, is the most common tick in Nigeria (Unsworth, 1952; Strickland, 1961; Mohammed, 1974).

In 1954, Hoogstraal reported Boophilus annulatus to be present in a British Museum collection of ticks from Nigeria, and in his extensive survey of the country Strickland (1961) found that this tick was widespread and reasonably common, though far less so than B. decoloratus.

Boophilus geigy was first described by Aeschlimann & Morel (1965) from West Africa. Mohammed (1974) and Dipeolu (1975) have found this species in small numbers in Nigeria.

The only other species of tick which has been shown to transmit B. bigemina, and which is also known to occur in Nigeria, is Rhipicephalus evertsi. In Nigeria this

tick is primarily a parasite of horses (Strickland, 1961). It is unlikely to be of any significance in the epidemiology of cattle babesiosis in Nigeria.

The present author made tick collections from cattle on the Jos Plateau in the neighbourhood of Vom. Boophilid ticks were found to be very common. The three species, B. decoloratus, B. annulatus and B. geigyi were present in the approximate ratio of 100 to 10 to 1, in a total of 1,250 boophilid ticks collected. Rhipicephalus evertsi was not found parasitising cattle, but was collected from a horse brought to Vom clinic.

a) The attempted isolation of Babesia bigemina from ticks

#### Materials and methods

Two splenectomised calves were used. No haemo-parasites had been observed in these animals during the frequent examination of blood smears for 28 days after splenectomy.

Engorging female Boophilus spp. ticks were collected from indigenous cattle which had been exposed to natural tick infestation throughout their lives. The ticks were identified as B. annulatus and B. decoloratus. Their larval progeny were fed on the two calves 30 days after emergence had been completed. Approximately 9,000 B. annulatus were applied to one calf and approximately 18,000 B. decoloratus were applied to the other. The day of application was designated day 0. The calves were kept in well separated pens in a special shed reserved

for tick feeding (see Materials and Methods) and were examined and bled each day from four days before, until thirty-five days after, the larvae were applied. In thin blood smears prepared on each of these days 10,000 red cells were examined.

### Results

Engorging nymphs were first apparent in the groin area of both calves on day 11 and, by day 17, fully engorged nymphae were moulting to adults. Between days 22 and 29, a total of 53 fully engorged female B. decoloratus (Plate 14) were collected from the floor of one of the tick-feeding pens. The modal engorgement time was 26 days. In the other pen, 31 B. annulatus females dropped, fully engorged, between days 25 and 28, with a mode of 26 days.

The calf infested with B. decoloratus did not become infected with any haemoparasite nor did it suffer a fever during the observation period.

B. annulatus apparently transmitted the spirochaete Borrelia theileri to the host calf. Parasites were seen in low numbers (0.03%)\* on days 18 and 19. A mild elevation of temperature to 39.5°C accompanied the patent parasitaemia. No other clinical symptoms were observed. A slight leucocytosis occurred on day 19.

\* - this parasite is of course extracellular, and the parasitaemia is the ratio of parasites/ $10^5$  erythrocytes expressed as a percentage.

Plate 14

Boophilus decoloratus ticks feeding on a calf. Females  
almost fully engorged - note also the small male.





b) The attempted isolation of B. bigemina from the blood of indigenous cattle

Materials and methods

Eight mls. of blood were collected from each of ten local cattle. The cattle were part of the Vom experimental herd and had been acquired from Fulani herdsmen on the Jos Plateau at various times during the preceding three months. None of the cattle was ill at the time of bleeding.

Thin smears and P.C.V. estimations were made from the blood of each animal. The blood was pooled, a thick smear was made from it and the blood was then injected intravenously into a splenectomised calf. (This calf had been found to be infected with Theileria mutans when first moved to the tick-proof quarters at two months of age. After it was splenectomised, no other haemoparasites had been seen in blood smears examined daily for 28 days after the operation).

Daily observations included the measurement of rectal temperature, the assessment of haematological parameters and the examination of thin blood smears stained with Giemsa and acridine orange. Occasional serum samples were also taken. Blood was frozen on day 10 as stabilate C.T.V.M. 14. Brain crush smears were made post-mortem.

Results

The P.C.V.s of the donor cattle were between 15% and 31%, and seven of these animals were found to be



infected with Theileria mutans. In the thick smear prepared from pooled blood, only T. mutans was observed.

At the time of inoculation, the calf was patently infected with T. mutans. After inoculation it developed patent parasitaemias of Babesia bigemina, Eperythrozoon teganodes and B. bovis. Details of their respective parasitaemias are given in Table 33. Passage of blood from this calf (described in the next section) revealed that it was also infected with Anaplasma marginale, Borrelia theileri and E. wenyoni.

The calf became febrile on day 6 and remained so until its death on day 12. By day 8, it was clinically ill and its plasma was noticeably icteric. By day 10, it was anorexic and very weak. On day 12, it was laterally recumbent and in opisthotonus. It was judged to be in extremis and was humanely destroyed.

Initially low (18%), the P.C.V. declined slightly in association with the B. bigemina parasitaemia and then more sharply in association with the rising B. bovis parasitaemia, reaching a nadir of 7% on the day of death. Immature erythrocytes were present in blood smears from day 10.

On post-mortem there was evidence of jaundice. In the renal pelvis and other parts of the body, fat was yellow in colour. The liver was pale and yellow. Urine in the bladder was red. The brain was diffusely pink. In brain crush smears, cerebral and cerebellar capillaries

## Discussion

Table 33

Details of parasitaemias of three haemoparasites observed in a splenectomised calf inoculated with blood from Nigerian cattle

	Prepatent period (days)	Day of max. parasitaemia	Maximum parasitaemia
<u>Babesia bigemina</u>	6	8	0.7%
<u>Eperythrozoon teganodes</u>	8	9	Not done
<u>B. bovis</u>	8	12	3.0%

were grossly dilated and were packed with B. bovis infected erythrocytes (Plate 15).

### Discussion

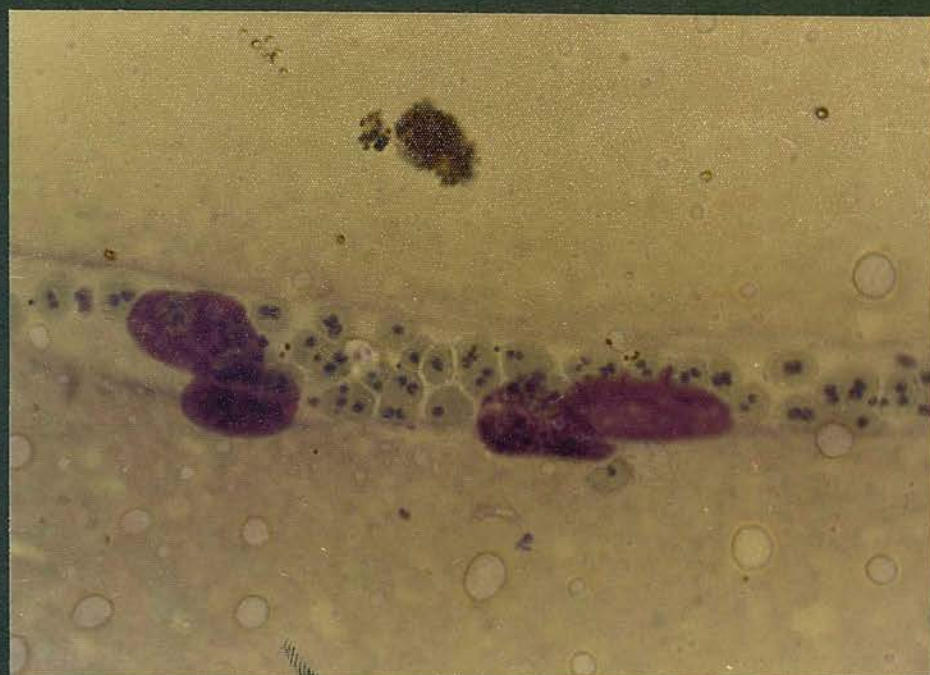
The collection, rearing and feeding of ticks is a more involved method of attempting to isolate B. bigemina than is the subinoculation of blood from latently infected cattle and in the present case it was not successful, although Borrelia theileri was apparently transmitted to the host calf. Leeflang (pers. comm.) regards Borrelia theileri as a common and non-pathogenic parasite of Nigerian cattle. In Brazil, it was shown by Brumpt (1919) to be transmissible by B. annulatus, but the finding in the present study appears to be the first African record of this vector-parasite relationship.

B. bigemina was successfully isolated by subinoculating blood from indigenous cattle into a splenectomised calf. In addition to B. bigemina, six other haemoparasites were isolated, although only T. mutans patently infected donor cattle. This compares with the results of Folkers & Kuil (1967) who splenectomised four Nigerian cattle and subsequently identified six different parasites in their blood. This and other unpublished work by Dutch parasitologists working at Zaria (P. Leeflang, pers. comm.), suggests that most Nigerian cattle are chronically infected with several haemoparasites. Precise data on the prevalence of haemoparasites in Nigeria are not available and serological surveys could reveal some

Plate 15

Babesia bovis in a brain crush smear - note the four endothelial cell nuclei and the dilated capillary packed with parasitised erythrocytes.





interesting findings in this respect since, although T. mutans patently infects the majority of local cattle, most other haemoparasites cannot be so readily observed.

The pathogenicity of B. bovis in Nigeria is not known. In the present instance, this organism was implicated in the death of a calf which exhibited symptoms of cerebral dysfunction. The pattern of decline in P.C.V. suggested that the B. bovis infection contributed substantially and lethally to the anaemia.

The purification of a Nigerian strain of B. bigemina

Having obtained a stabilate (C.T.V.M. 14) of B. bigemina (which contained other haemoparasites), six cattle were experimentally infected (see later section). At the same time, a series of experiments was set up in order to eliminate contaminants and to obtain a stabilate of pure B. bigemina. This was desirable for several reasons. Contaminating organisms might themselves be pathogenic, and so confuse an assessment of the pathogenicity of a Nigerian strain of B. bigemina. Even if they were not pathogenic, they might affect the normal course of B. bigemina primary parasitaemias. There is ample evidence that certain organisms will affect Babesia spp. in this way (Hoyte, 1961; Peters, 1965; Mahoney, 1972). It is possible that chronic parasitaemias and relapse behaviour could also be modified (Wright, 1971).

The method of purification was based on that described by Sergent, Donatien, Parrot, Lestoquard & Plantureux



(1927) and used by Callow & Hoyte (1961) and Bishop, Adams, Thompson & Corrier (1973). It involved the rapid passage of infected blood through a series of calves. In addition, selective chemotherapy was used to aid the elimination of certain contaminants where it was known that the drugs used would not affect B. bigemina.

#### Materials and methods

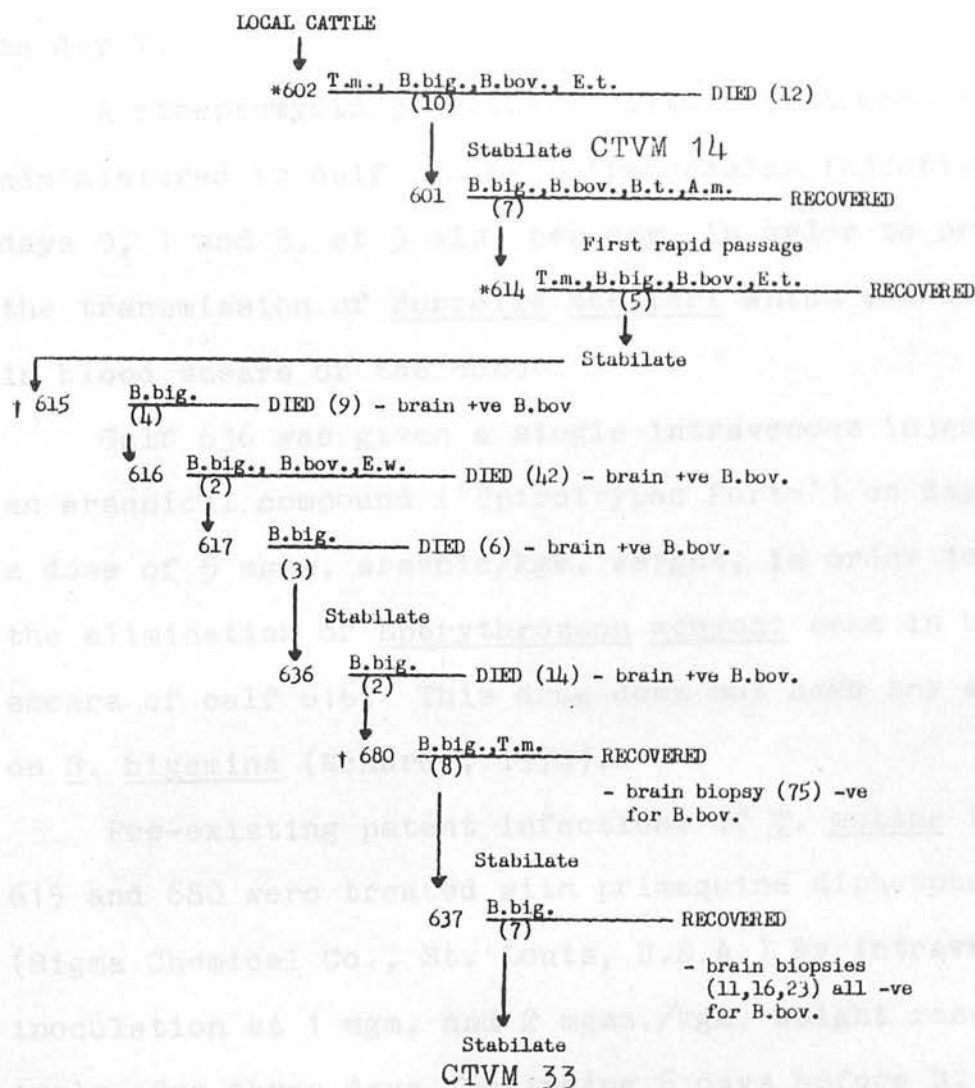
Eight calves were used, seven of which had been splenectomised. Three of these calves were naturally infected with Theileria mutans, but no other haemoparasites had been seen in blood smears from them taken frequently both after splenectomy and before experiment.

The passage series is illustrated in Fig. 22. After infection of the first calf with stabilate C.T.V.M. 14, blood was subinoculated, either directly as fresh blood or via preservation as stabilate, from one calf to the next on the first day that B. bigemina became patent in thin blood smears - with the exception of the sixth passage which was made 'blind' two days after calf 636 was inoculated. Blood smears were stained with acridine orange and  $10^5$  r.b.c.s were examined in each. Smears were prepared daily from the time of infection (day 0) until death or, in those calves which survived, for 20 days, after which smears were examined two or three times a week. Some smears were also prepared from blood obtained by pricking the tail-tip.

Brain crush smears were examined from post-mortem

Fig. 22

Schematic representation of the passage series employed to derive a pure isolate of Babesia bigemina from blood containing a mixture of haemoparasites.



Days of death, brain biopsy and subinoculation of blood indicated in parenthesis.

\* - Patently infected with Theileria mutans when B. bigemina inoculated.

† - Infected with T. mutans, but treated with primaquine, before B. bigemina inoculated.

Parasite abbreviations: B.big. - Babesia bigemina; B.bov. - B. bovis;

A.m. - Anaplasma marginale; T.m. - Theileria mutans; B.t. - Borrelia theileri;

E.t. - Eperythrozoon teganodes; E.w. - E. wenyonii.

N.B. - this figure includes the initial isolation, in calf 602, of B. bigemina, together with its subsequent purification by rapid passage through calves 601 to 637. Calf 616 died from the effects of E. wenyonii parasitaemia.

or biopsy material.

A stabilate (C.T.V.M. 33) was prepared from calf 637 on day 7.

A streptomycin/penicillin mixture ('Streptopen') was administered to calf 614 by intramuscular injection on days 0, 1 and 3, at 5 mls. per day, in order to prevent the transmission of Borrelia theileri which was observed in blood smears of the donor.

Calf 636 was given a single intravenous injection of an arsenical compound ('Spirotrypan Forte') on day 1, at a dose of 5 mgms. arsenic/kgm. weight, in order to aid the elimination of Eperythrozoon wenyoni seen in blood smears of calf 616. This drug does not have any effect on B. bigemina (McHardy, 1974).

Pre-existing patent infections of T. mutans in calves 615 and 680 were treated with primaquine diphosphate (Sigma Chemical Co., St. Louis, U.S.A.) by intravenous inoculation at 1 mgm. and 2 mgms./kgm. weight respectively, for three days, beginning 6 days before B. bigemina was passaged through them. By the third day of treatment T. mutans was no longer patent in blood smears.

### Results

The passage series is shown in Fig. 22. Parasites which were seen in blood smears are indicated on the horizontal lines, in the sequence in which they became patent.

It can be seen that in some cases the early death of

animals as a result of B. bigemina infection, prevented the development of patent parasitaemias of some organisms which were doubtless present, and which were seen in surviving calves of later passages. For this reason, it is not possible to say when certain contaminating organisms were eliminated. B. bigemina, B. bovis, T. mutans, Anaplasma marginale, Borrelia theileri, Eperythrozoon wenyonii and E. teganodes were all present in the original stabilate C.T.V.M. 14. A. marginale and B. theileri were eliminated, the latter with the aid of chemotherapy, by the first rapid passage. E. teganodes was not seen after the second passage but E. wenyonii was present in calf 616. T. mutans became patent in calf 680, 34 days after infection, but this parasitaemia was almost certainly a recrudescence of a pre-existing infection.

B. bovis was the most persistent contaminating organism. In calves which died without patent infection in the blood, brain crush smears prepared post-mortem were repeatedly positive for B. bovis. Because of this, the technique of brain biopsy was used to obtain brain crush smears from the last two surviving calves. A brain biopsy was taken on three separate occasions from calf 637 without its showing ill-effect. This calf naturally survived a B. bigemina parasitaemia, and blood smears examined from it for 75 days after infection remained free of haemoparasites with the exception of B. bigemina. The absence of other haemoparasites in blood and brain crush smears

is good evidence that the final stablitate (C.T.V.M. 33) contained only B. bigemina.

### Discussion

The number of passages required to obtain a pure infection of B. bigemina will partly depend on the level of patency adopted to detect it which will in turn determine the interval between passages. Using shorter passage intervals both Callow & Hoyte (1961) and Bishop, Adams, Thompson & Corrier (1973) obtained pure B. bigemina after four passages.

Most contaminating haemoparasites were quickly eliminated by rapid passage, and selective drugs such as penicillin for Borrelia theileri and arsenical preparations for Eperythrozoon spp. were useful adjuncts. The most awkward parasites to eliminate were Theileria mutans and B. bovis. The former presents difficulties for two reasons. Firstly, it naturally infects a high proportion of calves and, secondly, elimination therapy is not available. Three calves, which were born, reared and weaned indoors at Vom farm and then kept in tick-proof quarters, were found, when first examined at between 2 and  $3\frac{1}{2}$  months of age, to have patent T. mutans infections. The shortage of calves reared in tick-free surroundings was such that these T. mutans infected calves had to be used. Primaquine was found to be very useful for depressing parasitaemias prior to the passage of B. bigemina. Given before subinoculation, it did not appear to affect



B. bigemina. Passage of T. mutans was prevented by this stratagem, although the cure was not sterile and within a month or two T. mutans was once more patent in the treated calf. It was considered that this was a recrudescent parasitaemia in the light of similar results from other attempts - not described in this thesis - to treat T. mutans with primaquine.

The presence of T. mutans did not give rise to fears that the calves had been exposed to ticks. The probable route of infection was either intra-uterine or via biting insects. Although there is no indication in the literature that, in the case of T. mutans, either route occurs naturally (Barnett, 1968), other related Theileria spp. have been found infecting foetuses (Neitz, 1956).

B. bovis was not only persistent, but was also difficult to detect in blood smears, even though some smears were also made from tail-tip blood in view of the tendency of this parasite to concentrate in capillaries (Hoyte, 1971). The examination of brain crush smears was found to be a very real aid to detection and to be crucial in establishing with reasonable confidence that a calf was not infected with B. bovis. Calves tolerated the procedure of brain biopsy remarkably well.

Observations on the primary infection of cattle with a Nigerian strain of B. bigemina

a) Splenectomised calves

Materials and methods



These observations were made from five splenectomised calves infected serially during the purification of B. bigemina described previously. Only data from calf 615 onwards (see Fig. 22), i.e. the second rapid passage onwards, will be considered since other haemoparasites were not patent in these animals during the primary B. bigemina parasitaemia. It is possible that contaminating organisms still present at sub-patent levels affected the characteristics of the infections, but it is regarded as worthwhile to present the observations.

Each calf was infected with between  $10^5$  and  $10^6$  parasitised r.b.c.s.

### Results

All of the calves became infected, developed moderate to high parasitaemias and became clinically ill. Three calves died.

#### (i) Clinical symptoms

All the calves exhibited fever and haemoglobinuria and were anorexic for one or two days. One calf (636) developed secondary respiratory symptoms after the parasitaemic crisis. The animal went down on day 11, and developed catarrhal discharges from the eyes and nose. Dyspnoea was evident, and there were loud bronchial noises on auscultation. The calf died on day 14, six days after maximum parasitaemia. At post-mortem, the trachea, bronchi and bronchioles were filled with a yellow coloured froth.

Two other calves which died at the height of

parasitaemia, went 'down' a few hours before death. The two surviving calves recovered quickly without treatment.

(ii) The parasite

The normal appearance of parasites during rising parasitaemia is shown in Plate 8. Immediately following maximum parasitaemia, most parasites were shrunken and distorted in shape. Typical large paired organisms were rare. At this stage, some parasites were difficult to identify with confidence as being B. bigemina. After the parasitaemia had subsided, parasites were morphologically normal.

During the decline in primary parasitaemia and during the chronic parasitaemia, aggregates of parasitised erythrocytes were sometimes seen. This phenomenon varied from calf to calf, but in one of them was especially and frequently noticeable. Aggregates of extra-cellular parasites were also seen.

Brain crush smears from calves were examined for B. bovis (see previous section) at times when, coincidentally, B. bigemina was patent in blood smears. The latter parasite was seen only once in brain capillaries. It was morphologically normal and easily distinguishable from B. bovis.

(iii) Parasitaemia

Some parameters of the parasitaemias are presented in Table 34, together with data on the pyrexias and anaemias observed.

Table 34

Some parameters of parasitaemia, fever and P.C.V. in splenectomised calves infected with B. bigemina

	615	616	617	636	637
Prepatent period (days)	4	2	3	5	5
Day of maximum parasitaemia	7	5	6	8	7
First day of fever	6	3	4	8	6
Day of maximum fever	7	5	5	8	8
Duration of fever (days)	2	3	3	1	3
First day P.C.V. declined	7	5	6	8	8
Day of P.C.V. nadir	8	9	6	11	11
Day of death	9	-	6	13	-

The prepatent period was variable but short ( $3.8 \pm 1.3$  days). After patency, the growth rate was much the same in all the calves, and maximum parasitaemias were reached  $2.8 \pm 0.4$  days later. The development and course of parasitaemia was similar to that of B. divergens in splenectomised calves shown in Fig. 12, except that the rate of increase of B. bigemina parasitaemia was greater, and the rate of decline was slower, than the corresponding rates for B. divergens. The mean growth rate per day for the five calves during the phase of uniform logarithmic increase was a  $1.5 \pm 0.1 \log_{10}$  increase in parasitaemia per day. Recrudescences of parasitaemia were identifiable during the decline in parasitaemia of the two surviving calves. The mean relapse interval was  $4.5 \pm 1.3$  days.

The mean maximum percentage parasitaemia for the five calves was  $13.0\% \pm 11.8\%$  (range 2.4% to 31%).

#### (iv) Fever

The pyrexia occurred in association with higher parasitaemias (Table 34) and maximum fever tended to coincide with maximum parasitaemia. Mean maximum fever was  $41.1 \pm 0.4^{\circ}\text{C}$ . There was no correlation between the magnitude of maximum fevers and maximum parasitaemias.

#### (v) Anaemia

Pre-infection levels of P.C.V. ( $27.0\% \pm 3.2\%$ ) were stable up to the day of maximum parasitaemia, on which day they declined sharply by an average  $70.0\% \pm 11.8\%$  of pre-infection values. In survivors, the nadir

was reached four days after maximum parasitaemia, at about the same time as immature erythrocytes first appeared. Haemoglobinuria was observed in all the calves and was first seen on the day of maximum parasitaemia. Because the majority of the calves died, no attempt was made to correlate parameters of parasitaemia with those of P.C.V. depression.

(vi) Leucocytes

Changes in total leucocyte counts were neither as marked nor as consistent as those found in splenectomised calves infected with B. divergens, and the deaths of three of the calves limited the observations. The only consistent change was an absolute lymphocytopenia one or two days before maximum parasitaemia. This was in most cases accompanied by a similar degree of neutropoenia. In surviving calves, the lymphocyte count returned to normal in two or three days.

b) Non-splenectomised cattle inoculated with an impure isolate of B. bigemina

Materials and methods

Six steers, of between 20 and 23 months of age, were each infected with 0.5 ml. of thawed blood from stabilate C.T.V.M. 14. This stabilate was the original isolate of B. bigemina and also contained B. bovis, Anaplasma marginale, Eperythrozoon teganodes, E. wenyonii, Theileria mutans and Borrelia theileri.

Each animal was inoculated with approximately  $3 \times 10^6$

B. bigemina parasitised r.b.c.s and approximately  $4.5 \times 10^6$  B. bovis parasitised r.b.c.s. The day of infection was designated day 0. The cattle were bled from the coccygeal vein daily from day -2 to day 17, and then at intervals of three days to day 26. They were not then sampled until the first day of another experiment, (described later), 35 days after infection. The level of patency was 0.001%.

In order to check that the B. bigemina parasitaemia in coccygeal vein blood was no different from that in jugular vein blood, replicate smears from each source were made using blood from an infected animal, as has been described for B. divergens.

## Results

### (i) Clinical symptoms

With the exception of one animal (613), none of the cattle was anorexic or obviously ill, although 4/5 suffered a febrile reaction. Animal no. 613 developed a much higher parasitaemia than did the other five, became febrile and was seriously ill. By day 10 it was very weak and was lying down most of the time. It was anorexic and the faeces were firm but foul smelling, and were covered in blood and mucous. Haemoglobinuria was not observed. This animal recovered slowly without treatment.

### (ii) The parasite

Marked morphological changes did not occur during the course of reaction.



(iii) Parasitaemias

Four of the six cattle were found to be patently infected with T. mutans at the commencement of the experiment.

All six cattle became infected with B. bigemina. Patent parasitaemias of other organisms also developed in some of the cattle. Characteristics of their parasitaemias are shown in Table 35. None of these parasites established parasitaemias approaching in magnitude or duration those of B. bigemina. In addition, 35 days after infection, all six cattle were found to be infected with A. marginale.

Some parameters of the B. bigemina parasitaemias are shown in Table 36. The maximum parasitaemias were low except in one animal (613) in which parasitaemia reached a peak of 8.4%. The mean maximum percentage parasitaemia for the other five was  $0.040\% \pm 0.008\%$ . A period of uniform logarithmic growth was not observed. By the time that parasitaemias reached patent levels, the rate of increase of parasitaemia was declining daily.

There was no statistically significant difference between the mean parasitaemias derived from jugular and coccygeal vein blood ( $p = > 0.05$ ).

(iv) Fever

Some parameters of the fevers are shown in Table 36. The day of maximum fever coincided with the day of maximum B. bigemina parasitaemia. The mean maximum

Table 35

Some parameters of the parasitaemias of secondary agents in six non-splenectomised cattle infected with an impure isolate of *B. bigemina*

(v)	<i>Babesia bovis</i>	<i>Borrelia theileri</i>	<i>Eperythrozoon wenyoni</i>
No. of cattle with patent parasitaemias	4	6	2
Prepatent period (days)	Variable	$11.8 \pm 2.6$	17.0
Maximum parasitaemia %	$0.009 \pm 0.007$	$0.003 \pm 0.003$	Not done
Duration of parasitaemia (days)	$2.5 \pm 1.3$	$1.3 \pm 0.5$	1

Table 36

Some parameters of the *B. bigemina* parasitaemias and the fevers in non-splenectomised cattle infected with an impure isolate of *B. bigemina*

	603	605	608	610	611	613
Prepatent period (days)	7	6	6	8	7	5
Day of maximum parasitaemia	9	7	7	10	8	8
Duration of parasitaemia (days)	3	6	4	7	3	6
First day of fever	7	7	8	9	-	7
Day of maximum fever	9	7	8	10	-	8
Duration of fever (days)	3	1	1	2	-	2

temperature for the group was  $40.1 \pm 0.6^{\circ}\text{C}$ .

In one animal there was a transient pyrexia associated with a B. bovis parasitaemia.

(v) Anaemia

Red cell changes were not great, except in one animal (613), in which an 80% decrease of pre-infection P.C.V., to a nadir of 5%, occurred in association with the B. bigemina parasitaemia. In the other five animals, P.C.V.s declined during the course of the experiment. The rate of decline steepened in association with the B. bigemina parasitaemias and, in some cases, in association with succeeding B. bovis parasitaemias. For the five animals, the mean maximum depression in P.C.V. up to day 20, was  $32.0\% \pm 2.9\%$  of pre-infection levels. Immature erythrocytes appeared in three animals.

(vi) Leucocytes

Again, animal no. 613 reacted more severely than did the rest of the group, showing a profound leucopenia (nadir  $1.9 \times 10^3/\text{cu. mm.}$  from a pre-infection w.b.c. count of  $11.6 \times 10^3/\text{cu. mm.}$ ) associated with the B. bigemina parasitaemia. In the other five animals, there was a similar, though less extreme, decline in total leucocyte counts associated with the B. bigemina parasitaemias. The mean pre-infection leucocyte count of  $12.1 \pm 2.2 \times 10^3/\text{cu. mm.}$  fell to a nadir of  $6.4 \pm 1.0 \times 10^3/\text{cu. mm.}$  between 0 and 3 days after maximum parasitaemia. Thereafter, leucocyte counts quickly returned to near pre-

infection levels.

Differential counts showed that there was a uniform decrease in both lymphocytes and neutrophils during the leucopenia. The lymphocyte/neutrophil ratio remained unchanged, except in animal 613, in which there was a crossover in this ratio which persisted for two days.

c) Non-splenectomised cattle inoculated with a pure isolate of *B. bigemina*

Materials and methods

Ten steers, of between 20 and 32 months of age, were infected with stabilate C.T.V.M. 33, a stabilate of *B. bigemina* free from other haemoparasites. The inoculum per beast contained approximately  $10^8$  parasitised r.b.c.s. before freezing. This group was sampled daily for one week prior to infection and, as part of further experiments, for 64 days after infection. In this section, only results for the first 20 days after infection are presented and discussed. These animals were bled from the coccygeal vein.

Observations on the reaction of a single  $5\frac{1}{2}$  months old non-splenectomised calf (680) are also included. This calf was infected during the final stages of the purification of *B. bigemina* (see Fig. 22). It did not appear to have been simultaneously infected with any other haemoparasites. This calf harboured a natural infection of *T. mutans* which was treated with primaquine prior to this animal's infection with *B. bigemina*. The

inoculum dose of B. bigemina was not known, but was very small, since the parasitaemia in the donor was sub-patent at the time of subinoculation.

In addition to the usual observations, direct eosinophil counts were also carried out during the infection of the group of ten. Serum was collected from calf 680.

The day of infection was designated day 0. The level of patency was 0.001%.

### Results

When blood smears from the group of ten were first examined on day -7, nine were found to be patently infected with T. mutans but no other haemoparasite was observed. On day 0, after a week of daily sampling, three cattle were found to have low but patent parasitaemias of Anaplasma marginale. Without treatment, these parasitaemias subsided to sub-patent levels within a few days. The primary B. bigemina parasitaemias and ensuing reactions in these three animals were no different from those of the other seven cattle.

All the cattle became infected with B. bigemina and developed patent parasitaemias.

#### (i) Clinical symptoms

With the exception of fever in some animals, there were no signs of illness.

#### (ii) Parasitaemia

The primary parasitaemias for individual animals, together with subsequent induced relapse

parasitaemias, are shown in Fig. 25 (see later section). The chronological development of the primary parasitaemias in the group of ten was uniform, and data have been averaged. The prepatent period was  $5.3 \pm 0.7$  days and maximum parasitaemias ( $0.2\% \pm 0.2\%$ ) were reached  $7.3 \pm 1.2$  days after infection. The mean duration of parasitaemia was  $6.0 \pm 1.3$  days.

A phase of uniform logarithmic increase in parasitaemia was seen only briefly in some animals, before the rate of multiplication declined as parasitaemias reached maximum levels. From the limited data, the rate of uniform logarithmic growth varied from animal to animal, but lay between 0.5 and  $1.0 \log_{10}$  increase in parasitaemia per day. During the decline phase in the primary parasitaemia, a second distinct parasitaemic peak occurred in six animals. It was of lesser magnitude than the primary peak, and occurred, on average,  $3.2 \pm 1.0$  days after it. All parasitaemias were sub-patent by day 14 and remained so until day 20.

The primary B. bigemina parasitaemia was not affected by a preceding recrudescence of A. marginale.

The parasitaemia in the calf, 680, was very similar. The prepatent period was 8 days. Maximum parasitaemia of 0.19% was reached on day 10, and the parasitaemia quickly became sub-patent by day 12. A transient secondary patent parasitaemia reached a peak of 0.002% on day 15.



(iii) Fever

The calf 680 and four other animals exhibited fever. The mean maximum temperature for the five febrile animals was  $40.1 \pm 0.2^{\circ}\text{C}$  and the mean duration of fever was  $1.4 \pm 0.5$  days. The febrile zenith occurred on the same day as, or one day before, maximum parasitaemia. The mean maximum temperature observed for the group of ten was  $39.4 \pm 0.7^{\circ}\text{C}$ . The occurrence and duration of fever correlated positively with the level of maximum parasitaemia.

(iv) Anaemia

In the group of ten, P.C.V.s were affected by varying degrees. The maximum depressions in P.C.V., expressed as a percentage of pre-infection values, ranged from 0% to 33% with a mean of  $11.7\% \pm 9.6\%$ . The decline in P.C.V. was associated with the rise in B. bigemina parasitaemia as well as with its fall. Thus in one case, the nadir in P.C.V. occurred before maximum parasitaemia, though in the majority it occurred later (group mean time of P.C.V. nadir was  $1.3 \pm 1.3$  days after maximum parasitaemia). P.C.V.s quickly returned to pre-infection levels.

In the calf, 680, a P.C.V. depression of 18% of pre-infection levels occurred in association with the B. bigemina parasitaemia.

Haemoglobinuria was not observed in any of the cattle. Immature erythrocytes appeared in those three animals which suffered the greatest depression in P.C.V.

(a depression of 18% or above of pre-infection values).

There was a significant linear correlation between the magnitude of the depression in P.C.V. and the  $\log_{10}$  maximum parasitaemia ( $r = 0.88$ ,  $p = < 0.01$ ). Fig. 23 shows the regression for this relationship, with the line of best fit determined by the method of least squares.

(v) Leucocytes

Changes were not marked. A leucopenia occurred in five of eleven cattle in association with the B. bigemina parasitaemia. The leucocyte nadir occurred between 2 days before and 4 days after maximum parasitaemia and represented a maximum depression from pre-infection values of about 50%. In two of those most severely leucopenic, a crossover in the lymphocyte/neutrophil ratio occurred.

Direct eosinophil counts revealed fluctuating concentrations of eosinophils in those cattle with high counts. Counts in most animals rose after the commencement of sampling and then fell at the time of infection. In spite of fluctuations, a profound eosinopenia closely associated with the B. bigemina parasitaemia was pronounced in eight out of ten animals. The depression in eosinophil count was between 50% and 85% of pre-infection values and the nadir coincided, to within a day, with the maximum parasitaemia. Cattle with low pre-infection eosinophil counts did not suffer as profound a depression as cattle with high pre-infection counts.

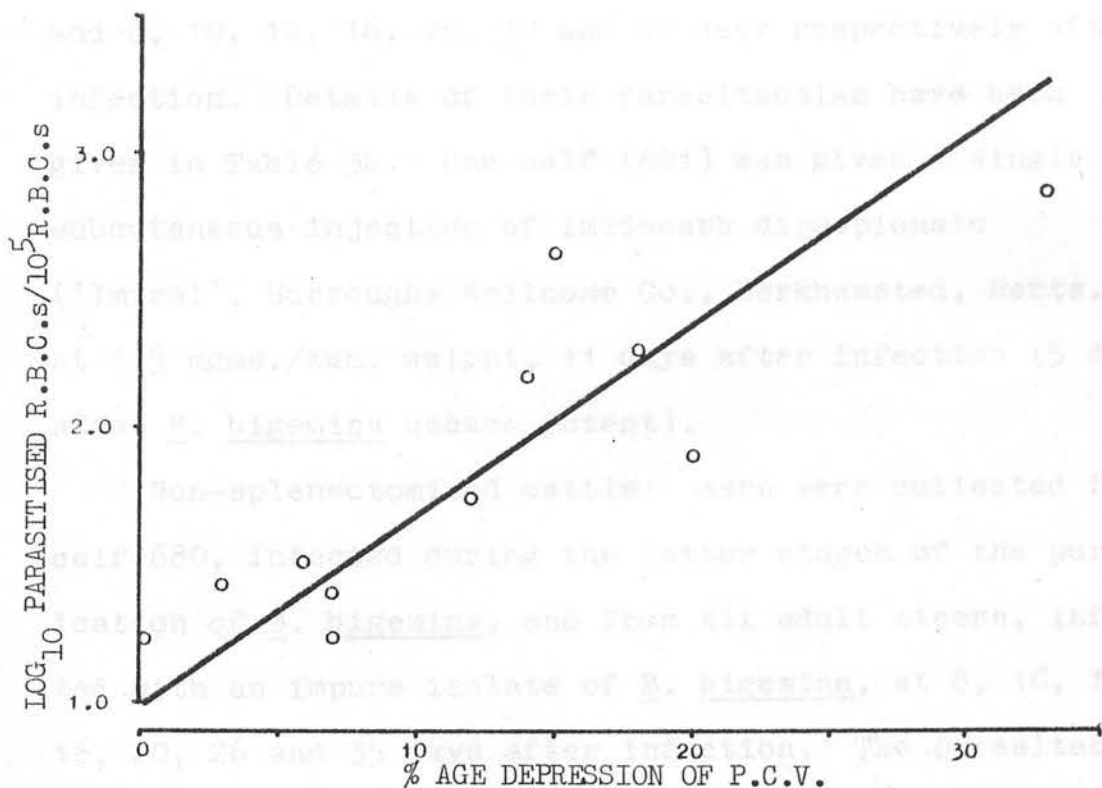


Fig. 23

The regression curve of maximum depression of P.C.V. (as a %age of pre-infection level) against maximum parasitaemia in B. bigemina infected non-splenectomised cattle.

d) The Indirect Fluorescent Antibody response

Materials and methods

Splenectomised calves: sera were collected during the infection of calves for the purification of B. bigemina. Serial samples were collected from two surviving calves, nos. 616 and 637, at 8, 11, 16, 25 and 40, and 8, 10, 12, 16, 25, 30 and 40 days respectively after infection. Details of their parasitaemias have been given in Table 34. One calf (601) was given a single subcutaneous injection of imidocarb dipropionate ('Imizol', Burroughs Wellcome Co., Berkhamsted, Herts.) at 1.5 mgms./kgm. weight, 11 days after infection (5 days after B. bigemina became patent).

Non-splenectomised cattle: sera were collected from calf 680, infected during the latter stages of the purification of B. bigemina, and from six adult steers, infected with an impure isolate of B. bigemina, at 8, 10, 12, 16, 20, 26 and 35 days after infection. The parasitaemias of these animals have been described previously and are illustrated in Table 36.

All sera were titrated against B. bigemina antigen at fourfold dilution intervals.

Results

Splenectomised calves

Of a total of eight splenectomised calves infected with B. bigemina with or without haemoparasites, four died. The times to death ranged from 6 to 13 days

after infection. Sera obtained at, or one day before, death were all negative. Of the four surviving calves, serum from one which was treated with imidocarb, was negative 30 days after infection. Sera from the three calves which survived without treatment were all subsequently positive to the I.F.A. test. Two of these calves, from which serial serum samples were collected, developed titres against B. bigemina between 8 and 12 days after infection. This was at least 3 days after maximum parasitaemia in each calf. Titres rose quickly to near maximum levels by 16 days after infection. Maximum titres were reached between 25 and 40 days after infection.

The geometric mean observed maximum titre for the three splenectomised calves was  $1/1,580$  (mean reciprocal  $\log_{10}$  titre  $3.2 \pm 0.2$ ).

#### Non-splenectomised cattle

The I.F.A. response of the calf did not differ from that in the adult steers.

All the cattle survived infection and developed titres against B. bigemina between 10 and 16 days after infection. In individual animals, this was a minimum period of 3 to 7 days after patency, and 1 to 5 days after maximum parasitaemia. Titres rose quickly to near maximum levels. The time of observed maximum titre varied from 16 to 35 days after infection.

The geometric mean maximum titre for the seven was  $1/575$  (mean reciprocal  $\log_{10}$  titre  $2.8 \pm 0.5$ ).

There was no significant difference between the mean maximum titres for splenectomised and non-splenectomised cattle ( $p = > 0.05$ ).

### Discussion

As was expected, splenectomised calves were far more severely affected by B. bigemina than were non-splenectomised cattle. Three splenectomised calves died and two survived. Working in E. Africa, Barnett (1965) expected 100% mortality in splenectomised calves, and Wright (1973) reports the death of 6/6 infected splenectomised calves in Australia. Working with a South African strain of B. bigemina, Löhr (1973) reported severe reactions in splenectomised Sahiwal cattle of various ages. Maximum parasitaemias ranged from 9% - 25%. Fever was a consistent symptom, but minimum P.C.V.s were not particularly low (20% - 49% - although Löhr later notes that Sahiwal cattle have a very high normal P.C.V. of between 50% - 80%). One animal died and the rest were either treated or killed for vaccine production. Löhr (1973) states that similar results were obtained with splenectomised Bos taurus type cattle.

In non-splenectomised steers the effects of infection were, with one exception, mild. The only observed symptom was a modest fever and even this was not consistently present. If the cattle had been part of a farm herd, it is doubtful whether their infection would have been noticed. The absence of frank disease, in cattle which



were substantially exotic in breeding, suggests that the strain of B. bigemina isolated in Nigeria was only mildly pathogenic.

Results obtained by Mahoney, Wright & Mirre (1973) from non-splenectomised adult Bos taurus steers which were infected with  $10^9$  B. bigemina parasites, are very similar to those obtained by ourselves. By comparison, when vaccinating Bos taurus type adult cattle with a low inoculum of B. bigemina ( $10^3$  parasites, Onderstepoort strain), Löhr (1969) reported severe reactions necessitating treatment in 50% of the cattle. He subsequently showed (Löhr, 1973) that adult Sahiwal cattle were not nearly so susceptible; after inoculation with  $10^7$  parasites (of the same strain), maximum parasitaemias of 0.05% - 0.8% occurred, there were no fevers and the mean fall in P.C.V. was 19%. These results accord well with our own findings, and raise the question of whether our cattle at Vom possessed an innate resistance due to their part Bos indicus breeding. The contribution of Bos indicus to the genotype of the cattle was always less than  $\frac{1}{2}$  and in most cases less than  $\frac{1}{8}$ . In the eleven cattle infected with a pure isolate of B. bigemina, those animals with the higher maximum parasitaemias (and hence greater P.C.V. depressions) tended to be the ones with the greater proportion of Bos taurus in the genotype. However, the range of genetic heterogeneity was small. Moreover, the most severe reaction of the group (in an

animal 31/32 parts Friesian) was, in absolute terms, a mild one.

The findings of Löhr (1969 and 1973) are in contradiction to those of Daly & Hall (1955), who found no difference in susceptibility to B. bigemina between British breeds and zebu-type cattle. These authors reported that about 30% of all cattle infected with B. bigemina as part of a vaccination programme required treatment. The other 70% had low parasitaemias and negligible clinical reactions.

The severity of reaction in calves inoculated with a purified Columbian isolate of B. bigemina varied according to the inoculum dose of parasites (Bishop & Adams, 1974). Inoculating calves, three months of age, with  $10^7$  parasites, these authors produced low parasitaemias (mean maximum 0.03%), modest pyrexias and a slight depression in P.C.V. Parasitaemias were higher and changes in temperature and P.C.V. more marked, when using an inoculum of  $10^9$  parasites. Using the same isolate and an inoculum dose of  $10^9$  parasites, Corrier & Adams (1973) obtained a mean maximum parasitaemia of 0.06% and a slight depression in P.C.V.

Comparisons of the pathogenicity of these isolates of B. bigemina from different parts of the world are complicated by the fact that the experiments are not usually directly comparable. The different studies quoted have used cattle of different ages and of different

genetic constitution, and the number of parasites inoculated has varied. Contrary to the observed field situations, age does not appear to affect the results of laboratory infection (several authors cited by Mahoney, 1972). However, the susceptibility of Bos taurus and of Bos indicus cattle may be different - as has been discussed - and the size of the inoculum probably has a profound effect on the severity of subsequent disease (Sergent, Donatien, Parrot, Lestoquard & Plantureux, 1927; Bishop & Adams, 1974; and in the case of B. divergens, Davies, Joyner & Kendall, 1958). Bearing these facts in mind, it appears that the strain of B. bigemina isolated in Nigeria resembles in pathogenicity strains from other parts of the world.

The results of laboratory infections by syringe inoculation cannot necessarily be extended to embrace natural tick induced infections. Apart from the influence on the parasite itself, natural challenge may involve exposure to other agents. To some extent, this effect has been reproduced by the laboratory infection of cattle with an impure isolate of B. bigemina. Whilst the results of this infection were mild, changes were more marked than those which occurred in similar cattle infected with a pure isolate of B. bigemina. The two groups are compared in Table 37. (The beast most severely affected by the impure isolate has been excluded from the calculation of the group means because its reaction was so exceptional).

Table 37

A comparison of infections produced by impure and pure isolates  
of B. bigemina in non-splenectomised steers

	Impure <u>B. bigemina</u> (n = 5)		Pure <u>B. bigemina</u> (n = 10)
Inoculum (parasitised r.b.c.s/beast)	$3 \times 10^6$		$10^8$
Maximum temperature °C	$40.1 \pm 0.6$	p = >0.05 N.S.	$39.4 \pm 0.7$
P.C.V. % age depression*	$32.0 \pm 2.9$	p = <0.01 Sig.	$11.7 \pm 9.6$
Maximum parasitaemia %	$0.04 \pm 0.008$	p = >0.05 N.S.	$0.16 \pm 0.25$

\* % age depression of pre-infection values

From Table 37, it can be seen that although the group infected with the impure isolate was inoculated with fewer parasites and suffered a lower mean maximum B. bigemina parasitaemia, it suffered a marginally higher pyrexia and a significantly greater depression in P.C.V. The sub-patent but rising presence of A. marginale probably contributed to the greater anaemia in this group (Corrier & Adams, 1973, have shown that the effects of concurrent B. bigemina and A. marginale are cumulative). Lower parasitaemias in this group probably reflect the inhibitory influence of T. mutans (Isihara, 1968; Mahoney, 1972) and Eperythrozoon spp. (Hoyte, 1961) although A. marginale has been found not to affect B. bigemina in this way (Corrier & Adams, 1973). This was confirmed by chance in our own experiments, when recrudescences of a pre-existing A. marginale parasitaemia coincided with the inoculation of B. bigemina in three cattle. Parasitaemias and reactions in these three cattle were no different from those in the other seven animals of the group.

The growth of parasitaemia resembled that observed for B. divergens, although in non-splenectomised cattle the  $\log_{10}$  phase of growth in the patent range was brief. In splenectomised calves, parasitaemia during the  $\log_{10}$  phase increased about half a  $\log_{10}$  increment/day faster than did B. divergens. This accounts for the short pre-patent periods observed. Relapse parasitaemias were seen immediately after primary parasitaemias. The mean relapse

interval was shorter than that observed in recovering B. divergens infected calves ( $4.5 \pm 1.3$  days compared with  $10.2 \pm 2.2$  days - the difference was significant,  $p = < 0.01$ ). Some non-splenectomised cattle also exhibited secondary parasitaemias and the mean relapse interval was again short ( $3.2 \pm 1.0$  days).

The development of anaemia in splenectomised calves, like that in B. divergens infections, was associated with the parasitaemic crisis, rather than with the rising parasitaemia. In contrast, data given by Wright (1973) shows that P.C.V. values fell substantially while the parasitaemia was rising. This tended to be the case in infections of non-splenectomised cattle which we observed. In his paper, Wright (1973) also presents the results of B. argentina infection in splenectomised calves and although details of the parasitaemias are scant, it appears that the observed decline in P.C.V. did not occur before maximum parasitaemias.

In the ten cattle infected with a pure isolate of B. bigemina, a range of maximum parasitaemia occurred naturally, and it was possible to correlate this parameter with the degree of anaemia. The regression curve (Fig. 23) cuts the 'y' axis at a parasitaemia of about 0.01%. This means that parasitaemias of less than this are unlikely to cause a fall in P.C.V. in non-splenectomised cattle.

As was stated in a previous discussion, the leucocyte



response to Babesia spp. infections is variable. It does not appear to be a reliable or useful parameter of infection. Eosinophil counts responded with reasonable consistency by falling to a nadir at maximum parasitaemia.

When blood smears from the group of ten cattle were first examined, in no animal was Anaplasma marginale patent. After one week, during which the cattle were bled daily, three animals had patent parasitaemias of this parasite. These parasitaemias were low and they quickly subsided, from which it was deduced that they were recrudescient and not primary parasitaemias. That three cattle should simultaneously suffer relapse parasitaemias a week after the commencement of observations is regarded as further evidence that the handling and bleeding of cattle can provoke recrudescences of latent haemoparasites. Eosinophil counts, conducted on alternate days, were not depressed by the commencement of handling the cattle.

The I.F.A. response to B. bigemina infection was similar to that observed to B. divergens. Calves which died did not develop antibodies before death. One calf was treated with imidocarb 11 days after infection. The dose used was sufficient to produce a sterile cure (Callow & McGregor, 1970) and this calf subsequently failed to develop antibodies. This result reaffirms the dangers of radical anti-babesial therapy during the course of the primary parasitaemia which Pipano, Weisman, Raz & Klinger

(1972) have shown will leave animals serologically negative and susceptible to future challenge.

Surviving calves did not have detectable levels of antibody in the blood until several days after maximum parasitaemia. Non-splenectomised cattle responded in a similar manner. The time of appearance of I.F. antibody was rather later than that reported for B. bigemina by Ross & Löhr, 1968, (2.1 days after the appearance of parasites) and by BurrIDGE, Kimber & McHardy, 1973 (7 days after infection). These workers used conjugated anti-bovine globulins, whereas, in our studies, a specific anti-IgG conjugate was employed. This fact may account for the discrepancy in results and, as has been noted in the case of B. divergens infections, it perhaps infers that, in bovine babesial infections, IgM antibody appears before IgG. On the other hand Goldman, Pipano & Rosenberg (1972) did not observe B. bigemina antibodies until 7-9 days after parasites were patent, although they were using a conjugated serum prepared against bovine globulins.

As was noted in the case of B. divergens, the absence of a spleen did not appear to affect the antibody response.

#### Observations on chronic B. bigemina parasitaemias and some factors which might influence them

##### a) Experiment 1

##### Materials and methods

Six non-splenectomised steers were used which had recovered, without treatment, from infection with an impure isolate of B. bigemina (described in an earlier section).

The animals were sampled daily from day 0, which was 35 days after infection. B-methasone was administered to three animals between days 62-65 and to the other three cattle between days 90-93. The untreated animals in each case served as controls. Observations ceased on day 115.

B-methasone was injected intramuscularly at 0.1 mgms./kgm. weight/day. Injections of normal saline were simultaneously given to control animals.

Oxytetracycline ('Terramycin' Q, Pfizer, Sandwich, Kent) was administered on different occasions in order to control Anaplasma marginale parasitaemias. A course of treatment consisted of one 10 mls. intramuscular injection /day for two days.

Sulphadimidine B.P. ('Sulphamezathine', I.C.I., Macclesfield, Cheshire) was given by mouth at the recommended dose for four days, to all six cattle, following the first series of B-methasone treatments.

Paired sera from each animal obtained before, and 23 days after, the first B-methasone treatments were submitted to Dr. W. Taylor of the Virology Dept., National Veterinary Research Institute, Vom, for examination for bovine virus diarrhoea (B.V.D.) antibodies by a serum neutralisation test.

Daily observations consisted of determinations of temperature, P.C.V.s and parasitaemias. Direct eosinophil, total leucocyte and differential leucocyte counts were carried out every 2-3 days between treatments and daily before, during and just after treatments.

### Results

Chronic B. bigemina parasitaemias in the individual animals are shown in Fig. 24. Also shown are Anaplasma marginale parasitaemias which were patent throughout much of this experiment. The first peak A. marginale parasitaemia shown is the primary parasitaemia which reached high levels 35-45 days after infection. In all animals, the primary parasitaemia of A. marginale caused an elevation in temperature and an anaemia, such that treatment with oxytetracycline was thought necessary to ensure the animals' survival and the continuance of the experiment. Up to the time of treatment, A. marginale had caused maximum temperatures of between  $39.1^{\circ}\text{C}$  and  $40.0^{\circ}\text{C}$  and depressions in P.C.V. of up to 75% of initial values. The treatment with oxytetracycline caused an immediate decline in A. marginale parasitaemias and a remission in clinical symptoms, but secondary parasitaemias later became patent. These subsided naturally and continued to fluctuate without treatment except in one animal (613) in which a second course of oxytetracycline was thought necessary.

It can be seen from Fig. 24 that during this period

Fig. 24

Chronic parasitaemias of B. bigemina (denoted by solid lines) in non-splenectomised cattle concurrently infected with A. marginale (denoted by dotted lines), and the effect of B-methasone administration. For other three cattle, see over.

B. BIGEMINA (PARASITISED R.B.C.s/ $10^5$  R.B.C.s)

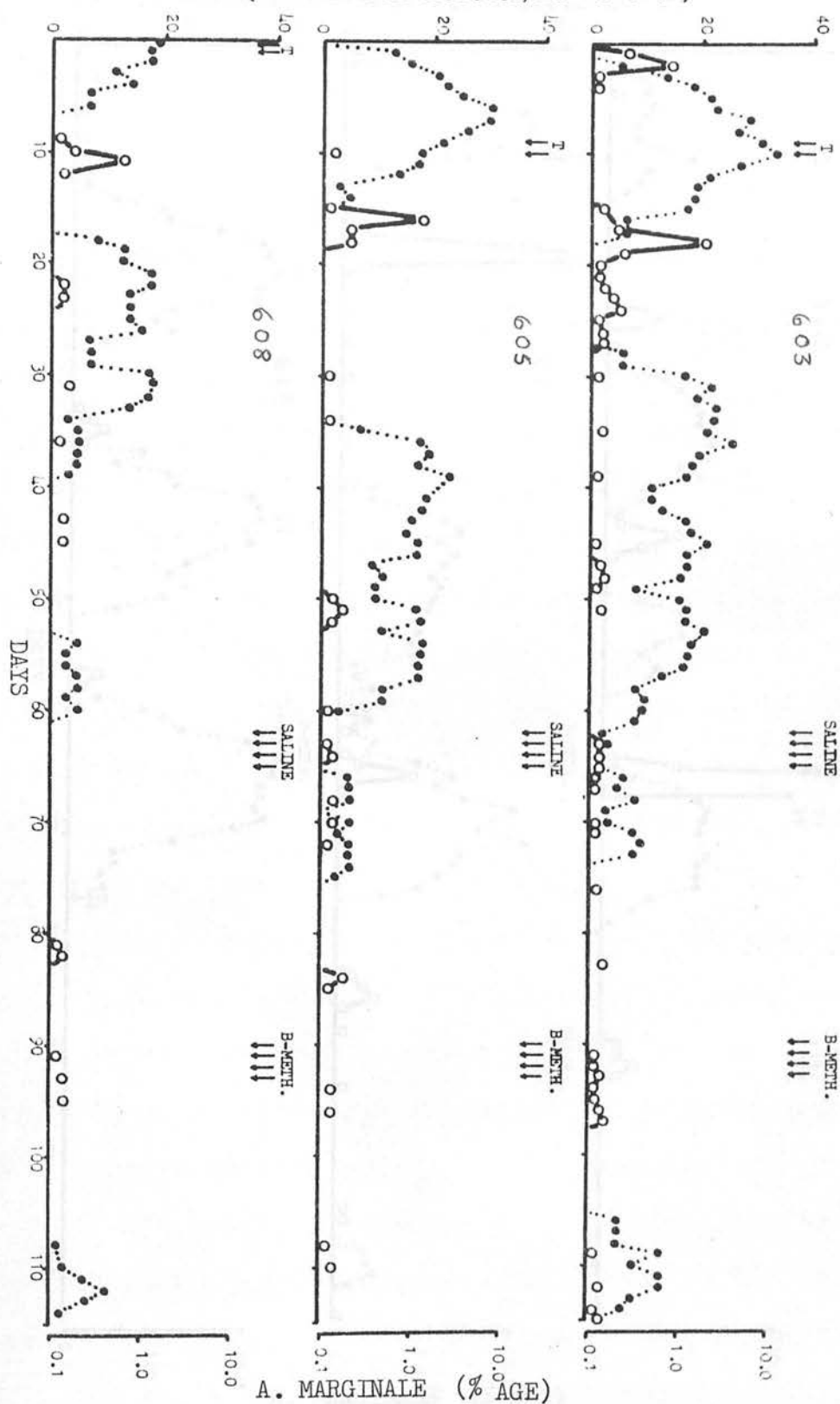


Fig. 24 (see opposite)



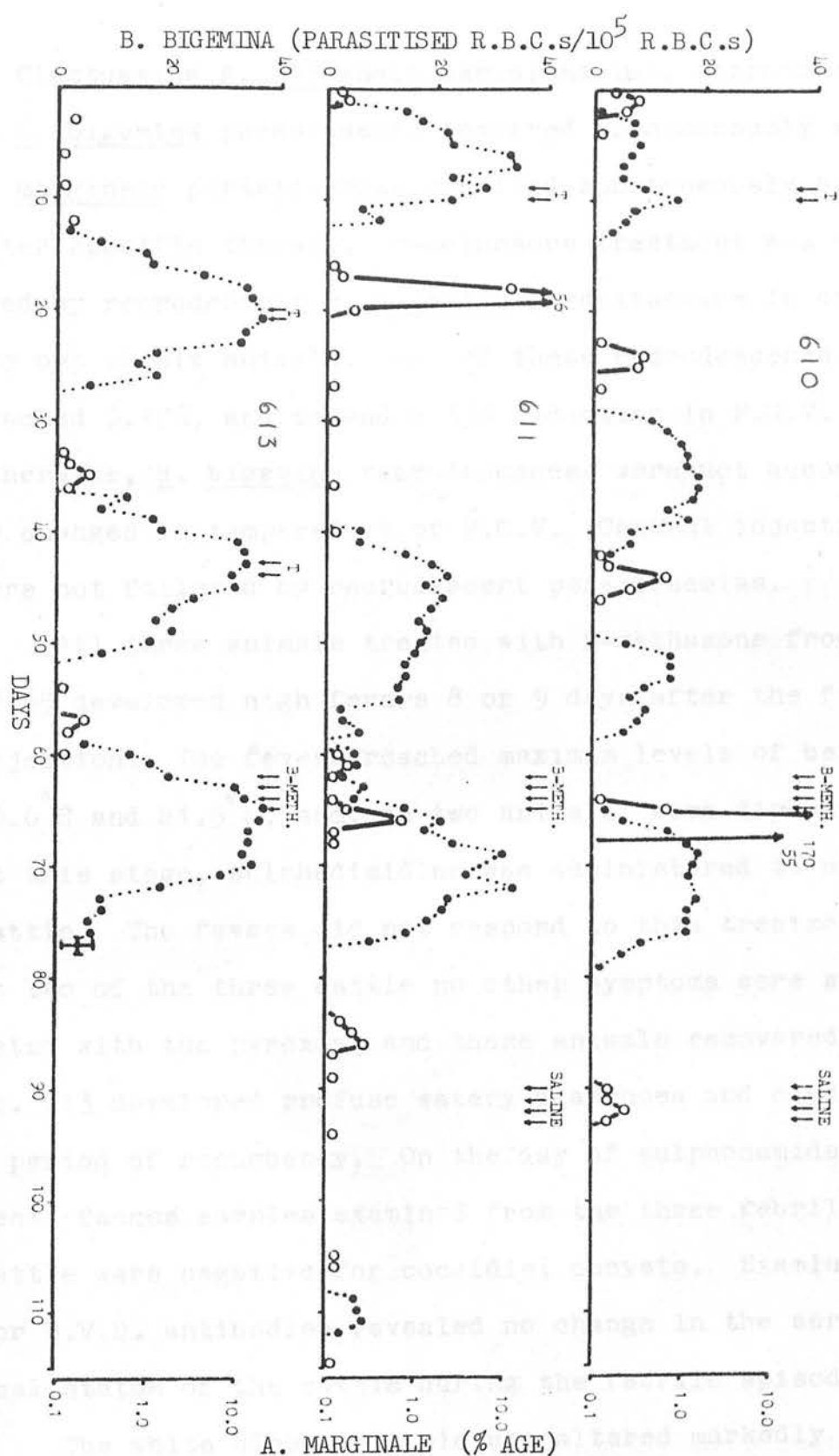


Fig. 24 (cont.)

of fluctuating A. marginale parasitaemias, recrudescences in B. bigemina parasitaemia occurred spontaneously when A. marginale parasitaemias subsided spontaneously or after specific therapy. B-methasone treatment was followed by recrudescence B. bigemina parasitaemias in only two out of six animals. One of these recrudescences reached 0.17%, and caused a 33% reduction in P.C.V. Otherwise, B. bigemina recrudescences were not accompanied by changes in temperature or P.C.V. Control injections were not followed by recrudescence parasitaemias.

All three animals treated with B-methasone from days 62-65 developed high fevers 8 or 9 days after the first injection. The fevers reached maximum levels of between  $40.6^{\circ}\text{C}$  and  $41.5^{\circ}\text{C}$ , and, in two animals, were diphasic. At this stage, sulphadimidine was administered to all six cattle. The fevers did not respond to this treatment. In two of the three cattle no other symptoms were associated with the pyrexia, and these animals recovered, but no. 613 developed profuse watery diarrhoea and died after a period of recumbency. On the day of sulphonamide treatment, faeces samples examined from the three febrile cattle were negative for coccidial oocysts. Examination for B.V.D. antibodies revealed no change in the serological status of the cattle during the febrile episode.

The white blood cell picture altered markedly following B-methasone treatment, but was unaffected by control injections. The results for all six cattle are

shown in Table 38. There was a leucocytosis, which comprised a massive neutrophilia, a lymphocytopoenia and an eosinopenia. At times other than when B-methasone was administered, spontaneous recrudescent B. bigemina parasitaemias were not associated with changes in the leucocyte picture.

Of the other haemoparasites known to be infecting these cattle, Theileria mutans was patent for most of the time in all of the animals. Parasitaemias rose slightly after B-methasone administration. Following corticosteroid administration, one pair of B. bovis parasites was seen in one animal. Between days 7 and 12 of the experiment, a primary parasitaemia of E. wenyoni was seen in another.

#### b) Experiment 2

##### Materials and methods

Ten non-splenectomised steers were used which had recovered, without treatment, from infection with a purified isolate of B. bigemina. These cattle were observed continuously from before infection until 64 days after infection. The primary parasitaemias and the accompanying reactions up to 20 days after infection have been described in an earlier section.

Times are measured from the day of infection, day 0.

Four-day treatments with B-methasone were carried out on 8 cattle in two series, as in previous experiments. The remaining two cattle were treated, by intramuscular

injection, with 2.5 ml. of 100:1 antigen suspension for four days.

Observations were conducted as before for experiment 1.

### Results

Primary and secondary *B. biguttatus* parasitemias are shown in Fig. 25. These results were found to be

Table 38

Total leucocyte and absolute differential counts in six cattle before and after B-methasone treatment  
(counts  $\times 10^3/\text{cu. mm.}$ )

*T. mutans*.

	Total leucocytes	Lymphocytes	Neutrophils	Eosinophils
Pre-treatment	$14.7 \pm 5.0$	$11.2 \pm 3.1$	$2.3 \pm 1.3$	$0.8 \pm 0.8$
	Sig. 5%	Sig. 1%	Sig. 1%	Sig. 5%
Post-treatment extremes	$26.1 \pm 9.0$	$3.5 \pm 1.8$	$19.6 \pm 6.6$	0

ment, recrudescence parasitemias were observed in all 5 treated cattle. They were of varying magnitude, but in 4 cattle were equal to, or greater than, the level of maximum primary parasitemia. In those two cattle with the smallest *B. biguttatus* recrudescences, *B. marginis* was concurrently patent during part of the treatment period. A.O.T.H. treatment was followed by transient patent *B. biguttatus* parasitemias of low magnitude. Control injections were not followed by recrudescence parasitemias.

The recrudescence parasitemias did not cause fever, but were accompanied by falls in P.O.V. and, in most

injection, with A.C.T.H. at 100 i.u./100 kgms. weight/day for four days.

Observations were conducted as described for experiment 1.

### Results

Primary and chronic B. bigemina parasitaemias are shown in Fig. 25. Three cattle were found to be chronically infected with A. marginale, and parasitaemias for this parasite are also shown in Fig. 25. During this experiment, nine of the cattle exhibited parasitaemias of T. mutans.

It can be seen from Fig. 25 that, with few exceptions, spontaneous recrudescent parasitaemias of B. bigemina did not occur before B-methasone treatment and that, for the most part, chronic B. bigemina parasitaemias were sub-patent. However, following B-methasone treatment, recrudescent parasitaemias were observed in all 8 treated cattle. They were of varying magnitude, but in 6 cattle were equal to, or greater than, the level of maximum primary parasitaemia. In those two cattle with the smallest B. bigemina recrudesences, A. marginale was concurrently patent during part of the treatment period. A.C.T.H. treatment was followed by transient patent B. bigemina parasitaemias of low magnitude. Control injections were not followed by recrudescent parasitaemias.

The recrudescent parasitaemias did not cause fever, but were accompanied by falls in P.C.V. and, in most

Fig. 25

B. bigemina primary and chronic parasitaemias in non-splenectomised cattle and the effects of B-methasone and A.C.T.H. administration. (Where it occurred, A. marginale parasitaemia is depicted by a dotted line). For other five cattle, see over.



B. BIGEMINA PARASITISED R.B.C.s/ $10^5$  R.B.C.s

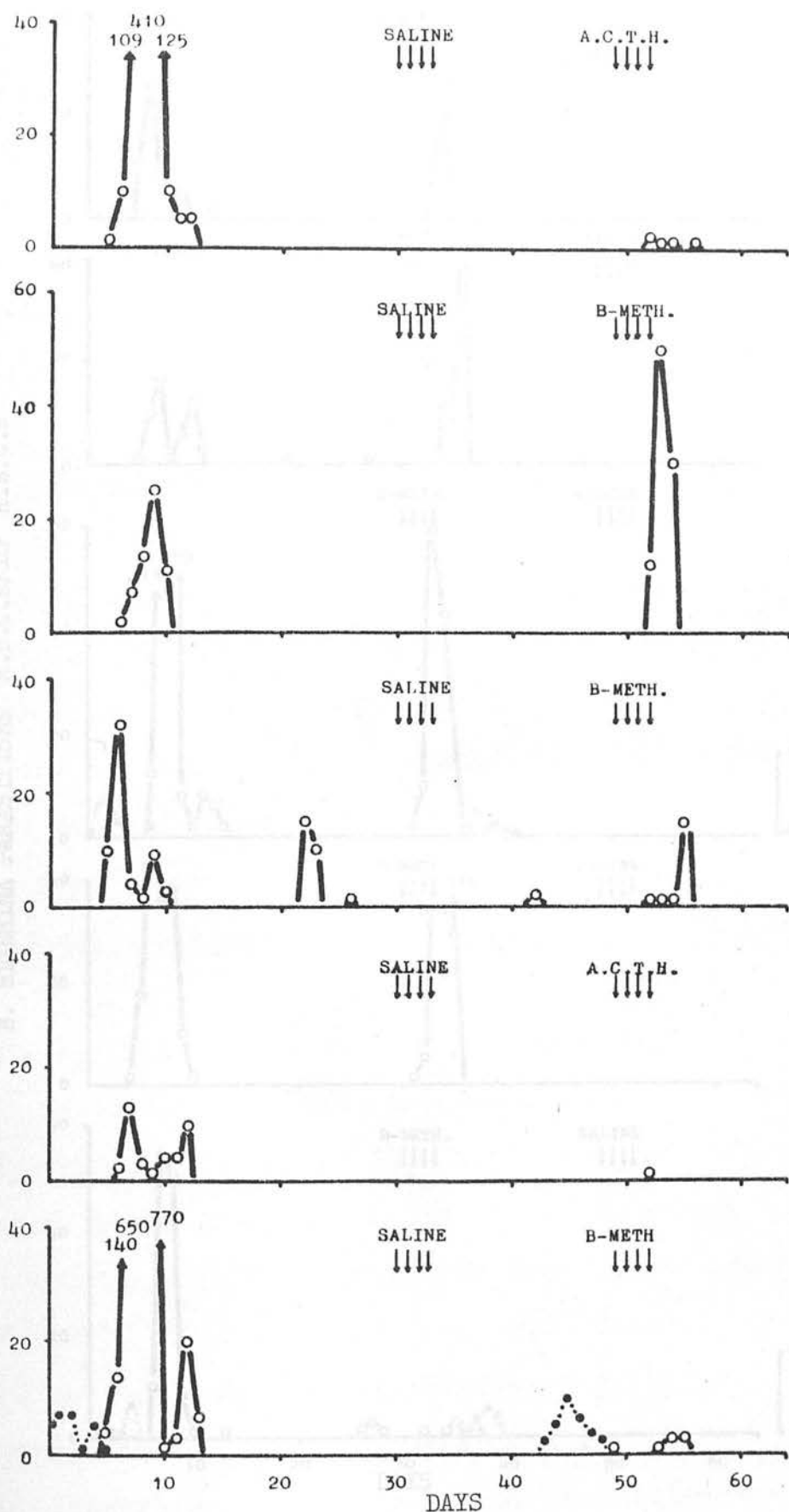
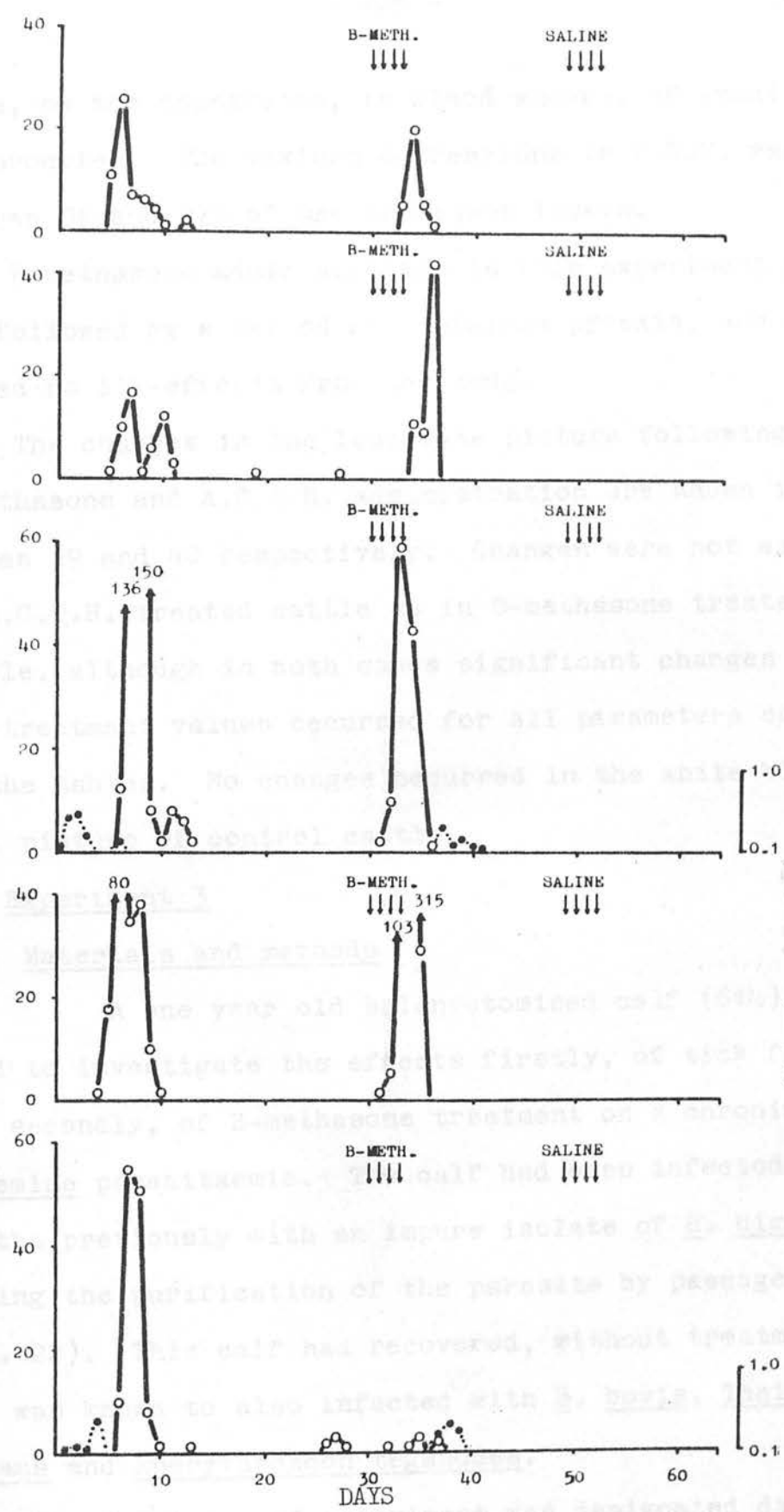


Fig. 25 (see opposite)

B. BIGEMINA PARASITISED R.B.C.s/ $10^5$  R.B.C.s



A. MARGINALE (% AGE)

Fig. 25 (cont.)

cases, by the appearance, in blood smears, of immature erythrocytes. The maximum depressions in P.C.V. ranged between 0% and 27% of pre-treatment levels.

B-methasone administration in this experiment was not followed by a period of sustained pyrexia, and cattle showed no ill-effects from the drug.

The changes in the leucocyte picture following B-methasone and A.C.T.H. administration are shown in Tables 39 and 40 respectively. Changes were not as severe in A.C.T.H. treated cattle as in B-methasone treated cattle, although in both cases significant changes from pre-treatment values occurred for all parameters compared in the tables. No changes occurred in the white blood cell picture of control cattle.

### c) Experiment 3

#### Materials and methods

A one year old splenectomised calf (614) was used to investigate the effects firstly, of tick feeding, and secondly, of B-methasone treatment on a chronic B. bigemina parasitaemia. The calf had been infected 4½ months previously with an impure isolate of B. bigemina during the purification of the parasite by passage (see Fig. 22). This calf had recovered, without treatment, and was known to also infected with B. bovis, Theileria mutans and Eperythrozoon teganodes.

The first day of experiment was designated day 0. On day 21, 22,000 mixed Boophilus decoloratus and B.

Table 39

Total leucocyte and absolute differential counts in eight cattle before and after B-methasone treatment  
(counts  $\times 10^3/\text{cu. mm.}$ )

	Total leucocytes	Lymphocytes	Neutrophils	Eosinophils
Pre-treatment	$15.8 \pm 5.0$	$11.3 \pm 4.6$	$3.2 \pm 1.5$	$0.3 \pm 0.1$
	Sig. 1%	Sig. 1%	Sig. 1%	Sig. 1%
Post-treatment extremes	$25.3 \pm 4.9$	$4.4 \pm 1.7$	$16.8 \pm 4.0$	$0.002 \pm 0.002$

Table 40

Total leucocyte and absolute differential counts in two steers before and after A.C.T.H. treatment (100 i.u./100 kgms. weight/day)  
(counts  $\times 10^3/\text{cu. mm.}$ )

	Total leucocytes	Lymphocytes	Neutrophils	Eosinophils
Pre-treatment	$13.5 \pm 0.1$	$7.2 \pm 0.1$	$3.0 \pm 0.7$	$0.6 \pm 0.1$
	Sig. 1%	Sig. 1%	Sig. 1%	Sig. 1%
Post-treatment extremes	$19.8 \pm 0.0$	$4.3 \pm 0.3$	$11.4 \pm 1.8$	$0.002 \pm 0.003$

annulatus larvae were applied. From earlier tick-feeding experiments, it was known that these ticks were not infected with Babesia spp.

After the completion of tick feeding, a four-day course of B-methasone (0.1 mgm./kgm. weight/day) was begun on day 54.

Observations were carried out as described for experiment 1. Serum was titrated for I.F. antibodies against B. bigemina on days 54, 57, 61, 64, 67 and 78. The experiment terminated on day 79.

Due to a pyrexia of unknown origin in the calf, sulphadimidine BP ('Sulphamezathine', I.C.I.) was administered orally from day 63 for five days.

Eight days after each tick had engorged, haemolymph smears were prepared by amputating the distal extremity of the second leg and smearing the extruded haemolymph on to a slide. Smears were stained by Giemsa's method.

### Results

The B. bigemina parasitaemia during the course of this experiment is shown in Fig. 26, together with a histogram illustrating the number of ticks which dropped, engorged, per day. Up to the time of B-methasone administration, the B. bigemina parasitaemia fluctuated at continuously patent levels with a definable periodicity. This pattern was not apparently influenced by the engorgement of ticks. From Fig. 26, six peaks of recrudescence parasitaemia can be identified up to day 52, and the mean

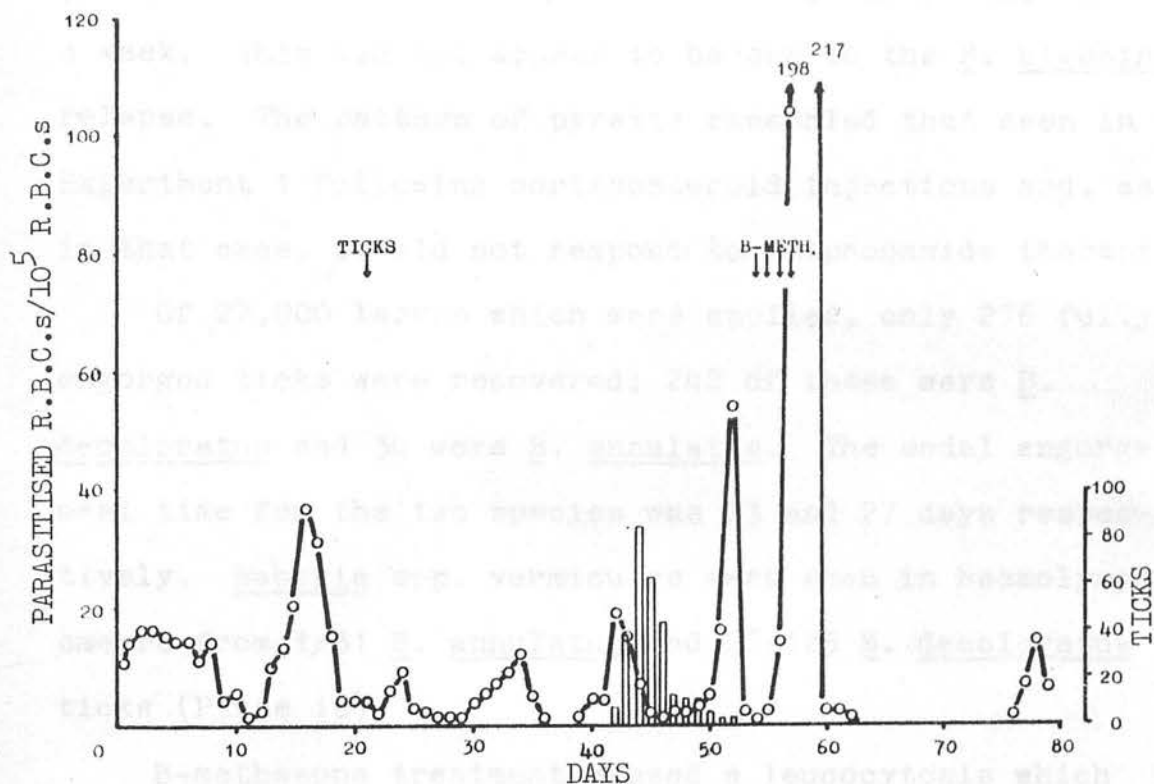


Fig. 26

The chronic B. bigemina parasitaemia in a splenectomised calf and the effect of tick feeding and B-methasone administration upon it. The histogram denotes the number of ticks which dropped engorged per day.



relapse interval is  $9.8 \pm 2.0$  days. B-methasone treatment provoked a recrudescent parasitaemia which reached 0.22%, and which was followed by a slight reduction in P.C.V. and the appearance of immature erythrocytes. A period of pyrexia followed B-methasone treatment, and persisted for a week. This did not appear to be due to the B. bigemina relapse. The pattern of pyrexia resembled that seen in Experiment 1 following corticosteroid injections and, as in that case, it did not respond to sulphonamide therapy.

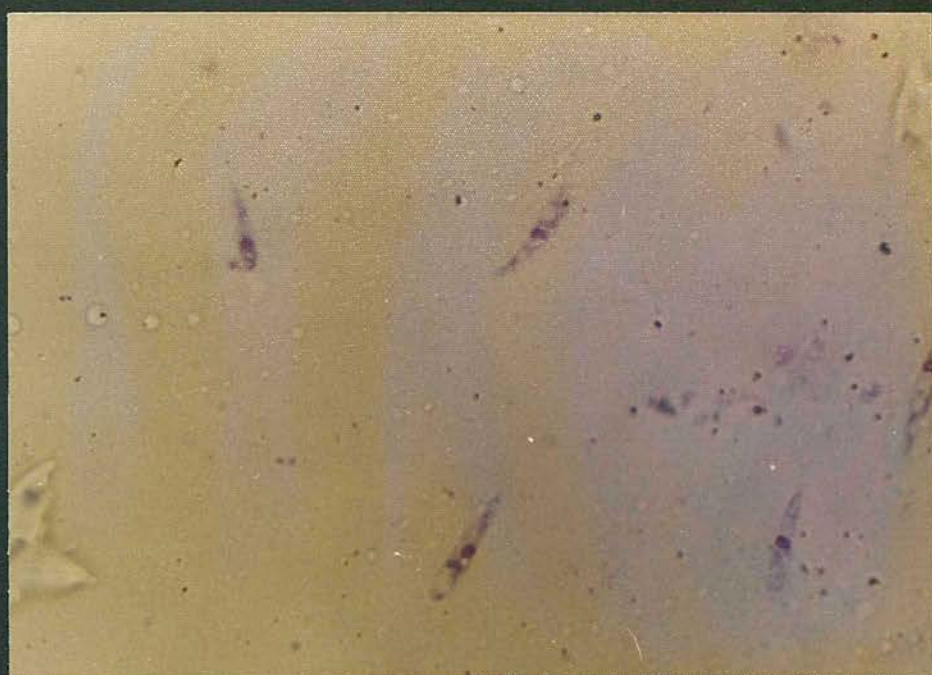
Of 22,000 larvae which were applied, only 276 fully engorged ticks were recovered; 242 of these were B. decoloratus and 34 were B. annulatus. The modal engorgement time for the two species was 23 and 27 days respectively. Babesia spp. vermicules were seen in haemolymph smears from 1/31 B. annulatus and 26/125 B. decoloratus ticks (Plate 16).

B-methasone treatment caused a leucocytosis which consisted of a lymphocytopenia (7.4 to 2.7), a neutrophilia (2.0 to 24.5) and an eosinopenia (0.04 to 0) - figures being respectively pre-treatment and post-treatment extreme counts  $\times 10^3/\text{cu. mm.}$  Neither the period of tick feeding nor the spontaneous recrudescences in B. bigemina parasitaemia was associated with changes in the white blood cell picture.

The I.F.A. titre at the commencement of B-methasone treatment was 1/160. No significant changes in titre were detected either during corticosteroid administration

Plate 16

Babesia bigemina vermicules in tick haemolymph





or following the recrudescent parasitaemia of B. bigemina.

### Discussion

In Experiment 1, relapse parasitaemias of B. bigemina occurred after natural or drug-induced falls in A. marginale parasitaemia. The reciprocal relationship was striking and consistent. The fact that relapse parasitaemias of B. bigemina did not (with rare exceptions) occur spontaneously in Experiment 2, suggests that B. bigemina recrudesences were actually provoked by a foregoing parasitaemia of A. marginale. In 1927, Sergeant, Donatien, Parrot, Lestoquard & Plantureux observed the same reciprocal relationship between A. marginale and B. bigemina. They gave it the delightful and appropriate name of 'occultation'. This phenomenon was not observed by Corrier & Adams (1973) in their studies of concurrent B. bigemina and A. marginale infections, in which parasitaemias of A. marginale persisted at high levels. The essence of 'occultation' is that recrudesences of B. bigemina will not occur whilst A. marginale is present at patent levels. Various reports of babesiosis relapses associated with anaplasmosis (Blood & Henderson, 1968; Ranatunga & Wanduragala, 1972) are probably manifestations of 'occultation'. Wright (1971) has described a similar situation existing between B. canis and Haemobartonella canis in which recrudesences of the two parasites alternated.

The recrudescent parasitaemias which were provoked

by A. marginale could not be associated with any consistent changes in eosinophil counts.

The results of Experiment 2 demonstrated clearly that recrudescent parasitaemias of B. bigemina could be provoked by B-methasone administration. The relapse parasitaemias were comparable in magnitude to the primary parasitaemias and caused a mild degree of anaemia in some animals. In the light of these results, the limited success of B-methasone in provoking recrudescent parasitaemias during Experiment 1 requires explanation. It can be seen from a study of Fig. 24, that, according to the phenomenon of 'occultation', a relapse of B. bigemina was to be expected in animal 610 at the time that B-methasone was administered. The corticosteroid, therefore, probably simply exacerbated this effect. The modest relapse in a second animal, and the absence of relapse in a third, simultaneously given B-methasone, are probably a result of an inhibitory effect by a concurrent patent parasitaemia of A. marginale. The absence of relapses later in the experiment, in three simultaneously treated cattle, is also possibly due to the presence of A. marginale at sub-patent but apparently inhibitory levels. In Experiment 2, there was also evidence that this parasite could inhibit the relapse response of B. bigemina, because the two animals with the lowest recrudesences following B-methasone treatment also showed A. marginale parasitaemias at about the same time.

The administration of A.C.T.H. was followed by very slight relapses of B. bigemina. It is possible that higher doses of A.C.T.H., which could have further raised endogenous corticosteroid concentrations, might have produced bigger recrudescence parasitaemias. In this respect, it is fruitful to examine the levels of plasma cortisol achieved in stress situations. Measurements of plasma cortisol in stressed cattle have been made after transport (Volkers, Furcht, Stolpe & Bauer, 1973), parturition (Heitzman, Adams & Hunter, 1970; Eberhart & Patt, 1971), exposure to high temperature (Christison, Mitra & Johnson, 1970) and over-milking (Paape, Desjardins, Schultze & Smith, 1972). These studies have shown that plasma cortisol usually increases by about twice pre-stress levels to between 5.0 and 9.0  $\mu$ gms./100 mls. and that elevated levels quickly return to normal after removal of the stressor. It has been shown by our own determinations in Edinburgh that a daily injection of A.C.T.H. at 100 i.u./100 kgms. weight, for four days, raised plasma cortisol to between two and three times that of pre-treatment levels for the duration of the drug administration. Since there is no reason to suppose that similar cattle in Vom responded differently (and the evidence of leucocytic changes confirmed this), it is evident that plasma cortisol levels were elevated by the same degree that certain stressors would have raised them. Furthermore, elevated cortisol levels were probably



maintained for rather longer than would have occurred following the application of the stressors which have been quoted.

Although only one calf was involved, the results of Experiment 3 establish some significant points. The calf was splenectomised and B-methasone treatment provoked a massive recrudescent B. bigemina parasitaemia. This demonstrates that the failure of B-methasone to provoke relapse parasitaemias in B. divergens infected splenectomised calves, compared with its success in provoking relapses of B. bigemina parasitaemias in many non-splenectomised cattle, was not related to the presence or absence of a spleen.

In the splenectomised calf, B. bigemina parasitaemias remained patent, and periodic fluctuations in parasitaemia were observed. The relapse interval averaged  $9.8 \pm 2.1$  days. This was shorter than the observed relapse interval in most B. divergens infected calves (see Table 29).

The engorgement of ticks did not affect the pattern of relapse parasitaemias. This experiment was prompted by the interesting results of Hoffman, Schein & Müller (1971) who, in both splenectomised and non-splenectomised calves, observed recrudescent parasitaemias of a chronic B. bigemina infection after the application of Boophilus spp. ticks. Furthermore, these workers apparently provoked relapse parasitaemias by the injection of tick salivary gland extracts. It is not clear to what extent

the effect of sampling and handling may have influenced these results but, if confirmed, they would obviously have considerable epidemiological significance.

The occurrence and effects of natural infection with B. bigemina in cattle around Vom

Observations

The assessment of the prevalence of any disease in indigenous cattle is extremely difficult because the herds are nomadic and veterinary services are as yet little utilised. It was possible, however, to obtain some impression of the incidence of clinical babesiosis from the work of Mr. N.B. Pullan, of the C.T.V.M. Project. As part of a wide-ranging study into factors limiting cattle production on the Plateau, he has established a close relationship, involving regular visits, with several settled Fulani herds comprising, in total, over 1000 cattle. I am grateful to Mr. Pullan for supplying the following information. In a period of more than a year, only one Fulani animal was observed to be clinically ill with babesiosis. This was a  $3\frac{1}{2}$  year old locally-bred bull, which suffered a maximum fever of  $41.6^{\circ}\text{C}$ , haemoglobinuria and a depression in P.C.V. to an observed nadir of 16%. The author examined blood smears from this animal on the second day of fever. The parasitaemic crisis appeared to have been reached, since parasites were morphologically abnormal. Whilst distinguishable as Babesia spp., they could not, with confidence, be further

identified.

Other observations were conducted on a herd of Friesian cattle kept at Vom Farm. These cattle are sprayed weekly with acaricide and are under veterinary supervision. From this herd, eleven cases of undiagnosed illness came to our notice during a period of 21 months. Whole blood or smears from these animals were supplied by the veterinary staff of the farm for our examination. One case of babesiosis was diagnosed. This involved a 2 year old cow born at Vom, which, without premonitory symptoms, suddenly collapsed and died. Blood smears prepared post-mortem contained abundant free Babesia spp. parasites. Erythrocytes were lysed and the morphology of the parasite was atypical probably due to a combination of some post-mortem degeneration and extensive pathological changes. The parasite in the blood was tentatively identified as B. bovis. A few parasitised erythrocytes were present in brain crush smears and, in this case, the parasite was more easily identifiable as B. bovis.

In January 1975, fifty in-calf heifers were imported to Vom from the United Kingdom. Their importation was seen as an unusual opportunity to determine by serological means if exotic sprayed cattle would become infected with B. bigemina, and to find out if clinical illness would accompany any conversions to a serologically positive status as might occur. Consequently, the cattle were bled on five occasions at regular intervals from February

1975 until March 1976. The sera were screened at 1/40 dilution and positive sera were also titrated.

Upon arrival in Nigeria, all the cattle were negative to the I.F.A. test for B. bigemina. After fourteen months at Vom, during which time they were kept out of doors, but sprayed at weekly intervals with acaricide, five animals had positive titres of 1/160 or above. The serological conversions occurred during, or shortly after, the rains (May - December). None of the cattle which developed positive B. bigemina titres was ill at any time during the fourteen months of the observation period. An examination of milking records showed that no falls in milk yield had occurred during the periods in which conversions occurred.

### Discussion

Five cattle became positive to the I.F.A. test for B. bigemina after their importation to Vom. The titres of positive reactors were not high but were comparable to those obtained in cattle experimentally infected with B. bigemina (see earlier section). These titres were higher than any likely to be obtained by cross-reactions with B. bovis or other agents (Ludford, 1969; Goldman, Pipano & Rosenberg, 1972; Leeftlang & Perié, 1972). The conversion of five cattle to a positive I.F.A. status against B. bigemina is therefore accepted as reasonable evidence that these animals became infected with the parasite.

The route of infection was probably via ticks. In spite of spraying, small numbers of ticks were observed feeding on cattle under the tail <sup>on the</sup> head and in other protected sites. Because the tick challenge was limited, presumably the B. bigemina challenge was similarly limited. Nevertheless, that natural tick-borne infection of susceptible cattle occurred, without clinical illness, is confirmation of the results of laboratory experiments that, in Nigeria, B. bigemina is not a highly pathogenic parasite.

The death, from babesiosis, of a cow at Vom farm provides further evidence that B. bovis can cause serious illness in Nigeria, although it is possible that the case described was a mixed infection of B. bigemina and B. bovis. In view of the fact that a proportion of Nigerian cattle harbour B. bovis in brain capillaries (Folkers, Kuil & Perié, 1967), the finding of modest numbers of parasites in the brains of sick or dead cattle cannot necessarily be regarded as an indication of the cause of illness or death.

The two natural cases described, highlight another problem of diagnosis. Whilst in the early stages of parasitaemia B. bigemina and B. bovis are easily distinguishable, at the time when parasitaemia is falling the author has found difficulty in categorically distinguishing between these two parasites. This difficulty may be compounded by such factors as the poor quality of smears

and staining which are associated with severely haemolysed blood and, in the case of cattle which die, by post-mortem degeneration, which in a tropical climate takes place very rapidly. Moreover, the period of falling parasitaemia coincides with the clinical crisis and is the time when the clinician is most likely to be required to identify parasites in blood smears. All these factors may combine to make it difficult in some cases of natural infection to ascribe the cause of illness or death to one Babesia spp. or the other although there should be little doubt that a Babesia spp. is responsible.

Failure to determine the cause. This was particularly serious in cases in Nigeria, where attempts to screen sera of indigenous cattle had to be abandoned. The problem was perhaps related more to the nature of the particular antigen preparation rather than to a fundamental limitation in the test itself, since Australian and other workers have successfully applied the I.F.A. test in field situations (Johansen, Pearson & Leitch, 1973; Mahler & Brown, 1974). By and large, the I.F.A. test was found to be a satisfactory means of detecting and measuring antibody, and is probably as efficient a means of so doing as any of the alternative serological tests presently available.

A high titre strain of B. biguttata was found to be only rarely pathogenic to non-splenectomized cattle infected experimentally. Furthermore, evidence has been



### CONCLUDING DISCUSSION

The I.F.A. test is frequently criticised because of the subjective opinion involved in determining the result. The author feels this criticism may be overstated. In experimental situations with B. divergens, titres were accurately reproduced in repeat tests. Moreover, most other serological tests also depend on a human assessment of some reaction such as agglutination. A more serious problem was found to be the occurrence of non-specific fluorescence, made all the more disconcerting by the failure to determine its cause. This was particularly serious in tests in Nigeria, where attempts to screen sera of indigenous cattle had to be abandoned. The problem was perhaps related more to the nature of the particular antigen preparation rather than to a fundamental limitation in the test itself, since Australian and other workers have successfully applied the I.F.A. test in field situations (Johnston, Pearson & Leatch, 1973; Mehlitz & Ehret, 1974). By and large, the I.F.A. test was found to be a satisfactory means of detecting and measuring antibody, and is probably as efficient a means of so doing as any of the alternative serological tests presently available.

A Nigerian strain of B. bigemina was found to be only mildly pathogenic to non-splenectomised cattle infected experimentally. Furthermore, evidence has been

presented that pure bred imported Bos taurus cattle were naturally infected with B. bigemina without ill-effect. The significance of the parasite does not, however, rest solely on its importance as a pathogen in its own right, but rather the combined effects of B. bigemina and other haemoparasites should be assessed in toto. At least in some circumstances the pathogenic effects of these parasites are cumulative (Corrier & Adams, 1973). Most Nigerian cattle are infected with five or six species of haemoparasite and in many cases it was noted that in the absence of frank disease or notable parasitaemias, the P.C.V. of native cattle was nevertheless abnormally low. The effect of this state of permanent sub-clinical anaemia on the growth and productive potential of domestic animals is surely significant.

Anaplasma marginale proved to be highly pathogenic to experimental animals and this parasite would appear to be a more serious threat to exotic cattle imported into Nigeria than Babesia spp. It alone would justify the application of strict acaricidal treatment to imported cattle in order to prevent mortality.

The role of B. bovis as a pathogen in Nigeria, and indeed throughout Africa as a whole, is unknown. Henning (1956), in an excellent account of babesiosis in South Africa, avoids any commitment to one Babesia spp. as prime cause of the disease. He cites Edington (1896) as the first to report B. bigemina as the cause of 'redwater' in

South Africa and this species has undoubtedly been frequently identified in association with the disease since that time. However, some pertinent features of the Australian babesiosis situation might apply equally to Africa. In Australia, where both B. bigemina and B. argentina are present, the latter organism is the major pathogen of the two (Johnston, 1968; Rogers, 1971a). Moreover, it frequently causes disease at very low parasitaemias (Legg, 1935). In view of the probable synonymy of B. argentina and B. bovis, one can see that the role of B. bovis in Africa could be underestimated. The report by Neitz (1941) that B. bovis was the cause of an outbreak of 'redwater' in Pretoria could be the tip of an aetiological 'iceberg'. Furthermore, the observation of Daly & Hall (1955) that Bos taurus breeds are more susceptible than Bos indicus cattle to B. argentina but not to B. bigemina, has the implication that exotic cattle imported to Africa may be threatened more by B. bovis than by B. bigemina.

The fact that the degree of red cell destruction which accompanies infections by Babesia spp. is frequently disproportionate to the parasitaemia has tended to obscure the fact that the degree of anaemia and clinical disease may nevertheless be correlated with the magnitude of parasitaemia. This correlation has seldom been observed, possibly because of the usually limited range of parasitaemia produced in experimental infections. Data have

been presented of both B. bigemina and B. divergens infections in which a range of parasitaemia naturally occurred which was large enough to demonstrate a positive correlation between maximum parasitaemia and anaemia. Similarly, Allen, Frerichs & Holbrook (1975) noted that in ponies there was a strong correlation between the degree of anaemia and the peak parasitaemia of B. caballi. Of course different Babesia spp. vary widely in their ability to cause disease at a given parasitaemia, but, within any one species (with the apparent exception of B. canis - Maegraith, Gilles & Devakul, 1957), it is probably true to say that a high parasitaemia will cause more profound host changes than a low one. In one experiment this relationship has been shown, over a limited range of parasitaemia, to be one of direct proportionality (Fig. 23 for B. bigemina). The construction of similar regression curves for each Babesia spp. would provide a most interesting means of comparing the pathogenicity of different species.

The degree of anaemia caused by a parasite is not, however, the sole criterion of its pathogenicity. Several times in this study the author has seen cattle survive amazingly low P.C.V.s (e.g. 5%) caused by various haemo-parasites. Conversely, many cattle which die of babesiosis do so just as the parasitaemia is falling, when the P.C.V. is probably not below that which can support life in certain circumstances. The contrast between the

pathogenic potential of similar parasites which cause a comparable degree of anaemia is no better illustrated than by that between B. rodhaini and B. microti in mice. At similar parasitaemias, these two parasites cause similar degrees of haemolysis (Gamble, 1974), and yet mice invariably survive B. microti infection and virtually always die from B. rodhaini infection.

That anaemia in babesiosis is not simply due to a destruction of red cells by parasites on a one for one basis has been known for some time, for various reasons mentioned in a previous discussion. The observation made with regard to B. divergens in splenectomised calves, in which haemolysis did not occur until the growth of parasitaemia was arrested, is further evidence that anaemia in babesiosis is not due to the physical effects of parasite invasion of erythrocytes. In non-splenectomised cattle infected with B. bigemina an association between haemolysis and parasitaemic crisis was not marked. The author suggests that a relationship between parasite death and haemolysis may still apply, but that its effect is not concentrated because parasite death begins earlier. This last statement is supported by a study of the growth of parasitaemia, which is seen to be slowing in non-splenectomised cattle almost as soon as it is patent. This, in turn, is possibly a result of a slightly earlier antibody response in non-splenectomised as opposed to splenectomised cattle. It has been shown that between non-splenec-



tomised cattle and splenectomised calves there was no difference in the ultimate size of antibody response. Ironically, this fact might be of considerable pathogenic significance. A strong antibody response in splenectomised calves coming at a time when high parasitaemias have been established, probably contributes to the destruction of the parasite population. This massive, sudden destruction of parasites may be a significant cause of the severe disease seen in splenectomised calves, analogous to the results of radical drug treatment noted by Sergeant, Donatien, Parrot, Lestoquard & Plantureux (1927). These workers were careful to point out that similar doses of drug (trypan blue) administered to chronically infected cattle did not produce toxic symptoms. In our own experiments, the association of parasite destruction with haemolysis does not prove a cause-effect relationship. The relationship may be coincidental and haemolysis may be primarily a result of other simultaneous antibody mediated factors, such as autoantibody or antibody directed against parasite antigen adsorbed on to erythrocytes. In support of the latter possibility, there is evidence that injected soluble antigens of B. rodhaini and B. canis will cause anaemia in normal rats and dogs (Sibinovic, Sibinovic, Ristic & Cox, 1967). In this respect it would be interesting to investigate the effects of the injection of large amounts of B. divergens breakdown products into normal calves.



If antibodies play a significant part in red cell destruction, immunosuppression during primary infection of splenectomised calves might lead to high parasitaemias but less severe anaemia. In a comparable situation, Clark & Allison (1974) infected hypothyroid mice with B. microti and observed high persistent parasitaemias which were not lethal. The situation is, however, not entirely analogous, since B. microti is not lethal in normal mice. One would like to know if the degree of anaemia suffered by hypothyroid mice is the same or less than that suffered by fully immunocompetent controls.

The effect of immunosuppression on chronic parasitaemias may also be paradoxical. It was certainly found to be variable. Only in some animals were recrudescent parasitaemias produced by B-methasone treatment. A number of factors may have contributed to this result. Study of the effect of immunosuppressants on primary antibody induction to a variety of antigens has shown that the timing of the immunosuppressant relative to antigen administration is critical for immunosuppression to be manifest (various authors cited by Bach, 1975). In these systems, the investigator controls the timing of both immunosuppressant and antigen administration. By contrast, the investigator has no control over the antigen factor in chronic infections, since variant antigen arises spontaneously as a product of the parasite's own antigenic lability. In our own experiments, it was noted that if

corticosteroid was coincidentally administered at times when a recrudescence was likely to occur e.g. after sampling, then the magnitude of recrudescence was exaggerated. Conversely, a 'timing' factor may explain the lack of response of B. divergens to corticosteroids. However, one would not have expected such consistently negative results if chance 'mis-timing' was responsible and, moreover, this explanation fails to take into account the uniform success of B-methasone in provoking recrudescences in some experiments involving B. bigemina.

The failure of B-methasone to provoke relapse parasitaemias in some cattle infected with B. bigemina appeared to be related to the concurrent presence of A. marginale. This parasite had two contrasting interactions with B. bigemina. At certain levels of parasitaemia it appeared to depress B. bigemina to the point of inhibiting a response to corticosteroid administration; on the other hand, a decline in A. marginale parasitaemia had a positive provocative effect on B. bigemina, an observation previously made by Sergeant, Donatien, Parrot, Lestoquard & Plantureux (1927).

If one dismisses the 'timing' explanation as being responsible for the lack of response of B. divergens parasitaemias to A.C.T.H. and B-methasone, one is initially tempted to presume that some other factor inhibited a response which might normally have occurred. The presence of an inhibitory factor (e.g. another parasite, such as

was observed in B. bigemina infections) is, however, unlikely. Apart from the fact that the experimental calves were only patently infected with B. divergens, spontaneous recrudescences in chronic parasitaemia did not appear to be inhibited in any way. Chronic parasitaemias of B. microti were also shown to be unresponsive to corticosteroid administration, an observation supported by the results of Young (1970). These findings suggest that Babesia spp. are inherently different in their response to corticosteroids. This does not seem likely, and the problem remains unresolved.

One of the most interesting results of the studies described, is the discovery that chronic B. divergens parasitaemias in splenectomised calves frequently and periodically relapse. In some cattle the parasitaemias were continuously patent so that minimum relapse intervals were observed and it has been suggested that this parameter may vary between different species of Babesia. The pattern of recrudescences is strongly suggestive of antigenic variation which has been shown to occur in B. rodhaini (Phillips, 1969a) and B. argentina (Curnow, 1973b). It remains to be proven that sequential recrudescences of B. divergens, such as were observed, consist of antigenically distinct variants.

Recrudescences in Babesia divergens (and Anaplasma marginale) parasitaemias were provoked by beginning to handle and sample animals and relapse parasitaemias of

B. bigemina appeared after A. marginale parasitaemias subsided. These recrudescences could not be correlated with marked changes in plasma cortisol concentration or leucocytic changes which might have reflected the latter. On the other hand, B. divergens parasitaemias in splenectomised calves were not affected by the elevation of plasma cortisol to levels, and for periods, equivalent to those which have been observed in cattle under stress (Christison, Mitra & Johnson, 1970; Heitzman, Adams & Hunter, 1970; Eberhart & Patt, 1971; Paape, Desjardins, Schultze & Smith, 1972; Volkers, Furcht, Stolpe & Bauer, 1973). These facts do not support the hypothesis that relapse parasitaemias following 'stress' are a direct result of raised endogenous corticosteroid levels, although in view of the various complicating factors which have been discussed, one would not be justified in rejecting the hypothesis. That handling provokes recrudescences in an experimental system supports the multitude of field observations which suggest that stress will cause relapse parasitaemias of Babesia spp. Whilst there is little doubt that elevated plasma cortisol levels would exacerbate this phenomenon, there is doubt from our findings that they alone could provoke recrudescences. In this respect other physiological sequels to 'stress' may also be significant. The effect of adrenalin would be worth investigating, although one might argue that its presence is too transient to have an effect. Nevertheless,

adrenalin has been shown experimentally to re-activate latent virus infections (Plummer, Hollingsworth, Phuangsab & Bowling, 1970).

It is clear that there are many complex factors which influence the host-parasite balance which pertains during chronic Babesia spp. parasitaemias. The effect of corticosteroids is less consistent than may have been anticipated. Studies on factors which influence chronic Babesia spp. parasitaemias, as well as having significance in their own right, could have relevance to other haemoparasitic diseases and to a much wider field beyond, since many infectious agents are characterised by persistent latent infections (Sergeant, 1963). In view of the increasing awareness that disease is in many cases a product of an imbalance in a pre-existing host-agent relationship rather than the infection of a naive host, studies of factors which affect the equilibrium between host and parasite are highly pertinent.

I am grateful to Drs. J.K.H. Wilder, A.R. Gray and G.R. Scott for their readiness at all times to answer questions and provide advice, and to other colleagues at the C.T.V.W., too numerous to mention individually, whose friendship and assistance has made this work so pleasurable.

I would also like to thank Dr. B.M.A. Latif for his useful advice during the setting up of the I.P.A. test.

### ACKNOWLEDGMENTS

The studies described in this thesis were funded by grant R2528 of the Ministry of Overseas Development, whose financial support is gratefully acknowledged.

I am grateful to Professor Sir Alexander Robertson for the provision of excellent facilities at the C.T.V.M., and to him and Dr. D. Branagan for their supervision of this study. In particular, Dr. Branagan's criticism during the preparation of this thesis has been invaluable.

I would like to thank the director (Dr. Musa Goni and latterly Dr. K.A.O. Sansi) and staff of the National Institute for Veterinary Research, Vom, for their generous provision of facilities and for their kind assistance during my stay in Vom. I am also indebted to Mr. D.F. Laing, Project Co-ordinator, C.T.V.M./Vom Collaboration Project, for his help in many ways in Nigeria.

I am grateful to Drs. J.K.H. Wilde, A.R. Gray and G.R. Scott for their readiness at all times to answer questions and provide advice, and to other colleagues at the C.T.V.M., too numerous to mention individually, whose friendship and assistance has made this work so pleasurable.

I would also like to thank Dr. B.M.A. Latif for his useful advice during the setting up of the I.F.A. test.



REFERENCES

- AESCHLIMANN, A. & MOREL, P.C. (1965). 'Boophilus geigyi n. sp. (Acarina: Ixodoidea) une nouvelle tique du bétail de l'Ouest Africain'. Acta Tropica, 22, 162-168.
- AESCHLIMANN, A. & SUTER, H. (1965). 'A propos de Babesia rodhaini'. Acta Tropica, 22, 303-320.
- ALLEN, Patricia C., FRERICHS, W.M. & HOLBROOK, A.A. (1975). 'Experimental acute Babesia caballi infections'. Experimental Parasitology, 37, 67-77.
- ANONYMOUS (1976). 'Human babesiosis'. Lancet, Vol. I of 1976, 1001-1002.
- BABES, V. (1888). 'Sur l'haemoglobinurie bactérienne du boeuf'. Compte rendu hebdomadaire des séances de l'Académie d'agriculture de France, Paris, 107, 692-694.
- BACH, J.F. (1975). 'The mode of action of immunosuppressive agents'. North Holland Publishing Co., Amsterdam and Oxford.
- BARNETT, S.F. (1961). 'The Control of Ticks on Livestock'. F.A.O. Agricultural Studies, no. 54, F.A.O., Rome.
- BARNETT, S.F. (1965). 'The chemotherapy of Babesia bigemina infection in cattle'. Research in Veterinary Science, 6, 397-415.

- BARNETT, S.F. (1968). In 'Infectious Blood Diseases of Man and Animals' (Weinman, D. & Ristic, M., eds.) Vol. 2, pp. 269-328. Academic Press, New York and London.
- BARNETT, S.F. (1974). 'Economical aspects of tick-borne diseases control. I. In Britain'. Bulletin de l'Office International des Epizooties, 81, 167-182.
- BASSETT, J.M. & HINKS, N.T. (1969). 'Micro-determination of corticosteroids in ovine peripheral plasma: effects of venipuncture, corticotrophin, insulin and glucose'. Journal of Endocrinology, 44, 387-403.
- BERGHE, L. van den, VINCKE, I.H., CHARDOME, M. & BULKE, M. van den (1950). 'Babesia rodhaini n. sp. d'un rongeur du Congo Belge transmissible à la souris blanche'. Annales de la Société Belge de Médecine Tropicale, 30, 83-86.
- BEVERIDGE, Elizabeth (1953). 'Babesia rodhaini: a useful organism for the testing of drugs designed for the treatment of piroplasmosis'. Annals of Tropical Medicine and Parasitology, 47, 134-138.
- BISHOP, J.P. & ADAMS, L.G. (1973). 'Combination thick and thin blood films for the detection of Babesia parasitaemia'. American Journal of Veterinary Research, 34, 1213-1214.

- BISHOP, J.P. & ADAMS, L.G. (1974). 'Babesia bigemina: immune response of cattle inoculated with irradiated parasites'. Experimental Parasitology, 35, 35-43.
- BISHOP, J.P., ADAMS, L.G., THOMPSON, K.C. & CORRIER, D.E. (1973). 'The isolation, separation and preservation of Babesia bigemina'. Tropical Animal Health and Production, 5, 141-145.
- BLOOD, D.C. & HENDERSON, J.A. (1968). 'Veterinary Medicine'. 3rd. Ed., Baillière, Tindall and Cassell, London.
- BRANAGAN, D. (1965). 'Observations on post-vaccinal sequelae to rinderpest vaccination using caprinised vaccine, on cattle in Tanganyika Masailand'. Bulletin of Epizootic Diseases of Africa, 13, 5-10.
- BROCKLESBY, D.W., HARNESS, E. & SELLWOOD, S.A. (1971). 'The effect of age on the natural immunity of cattle to Babesia divergens'. Research in Veterinary Science, 12, 15-17.
- BROWN, K.N. & BROWN, I.N. (1965). 'Immunity to malaria: antigenic variation in chronic infections of Plasmodium knowlesi'. Nature, (London), 208, 1286-1288.
- BROWN, I.N., BROWN, K.N. & HILLS, L.A. (1968). 'Immunity to malaria: the antibody response to antigenic variation by Plasmodium knowlesi'. Immunology, 14, 127-138.

- BROWN, K.N., BROWN, I.N. & HILLS, L.A. (1970). 'Immunity to malaria. I. Protection against Plasmodium knowlesi shown by monkeys sensitised with drug-suppressed infections or by dead parasites in Freund's adjuvant'. *Experimental Parasitology*, 28, 304-317.
- BRUCE-CHWATT, L.J., DORRELL, J. & TOPLEY, E. (1972). 'Antilymphocyte serum and the course of rodent malaria in mice'. *Transactions of the Royal Society for Tropical Medicine and Hygiene*, 66, 522-523.
- BRUMPT, E. (1919). 'Existence de la Spirochétose des Bovidés au Brésil. Transmission de cette affection par la tique: Margaropus australis (Fuller)'. *Bulletin de la Société de Pathologie Exotique*, 12, 748-757.
- BÜNGENER, W. (1967). 'Die Vermehrungsgeschwindigkeit von Plasmodium berghei, Plasmodium vinckei, und Babesia rodhaini'. *Zeitschrift für Tropenmedizin und Parasitologie*, 18, 463-466.
- BURRIDGE, M.J., KIMBER, C.D. & McHARDY, N. (1973). 'Detection of antibodies to Babesia bigemina in dried blood samples using the Indirect Fluorescent Antibody test'. *Annals of Tropical Medicine and Parasitology*, 67, 191-195.
- CALLOW, L.L. (1964). 'Strain immunity in babesiosis'. *Nature*, (London), 204, 1213-1214.

- CALLOW, L.L. (1967). 'Sterile immunity, co-infectious immunity and strain differences in Babesia bigemina infections'. Parasitology, 57, 455-465.
- CALLOW, L.L. & HOYTE, H.M.D. (1961). 'The separation of Babesia bigemina from Babesia argentina and Theileria mutans'. Australian Veterinary Journal, 37, 66-70.
- CALLOW, L.L. & JOHNSTON, L.A.Y. (1963). 'Babesia spp. in the brains of clinically normal cattle and their detection by the brain smear technique'. Australian Veterinary Journal, 39, 25-31.
- CALLOW, L.L. & MCGREGOR, W. (1970). 'The effect of imidocarb against Babesia argentina and Babesia bigemina infections of cattle'. Australian Veterinary Journal, 46, 195-200.
- CALLOW, L.L., MCGREGOR, W., PARKER, R.J. & DALGLIESH, R.J. (1974). 'The immunity of cattle to Babesia argentina after drug sterilisation of infections of varying duration'. Australian Veterinary Journal, 50, 6-11.
- CALLOW, L.L. & PARKER, R.J. (1969). 'Cortisone-induced relapses in Babesia argentina infections of cattle'. Australian Veterinary Journal, 45, 103-104.
- CANNON, W.B. (1929). 'Bodily changes in pain, hunger, fear and rage'. 2nd. Edition, C.T. Brandford Co., Boston.

CHRISTISON, G.I., MITRA, R. & JOHNSON, H.D. (1970).

'Glucocorticoids in acutely heat stressed steers'.

Journal of Animal Science, 31, 219.

CLARK, I.A. & ALLISON, A.C. (1974). 'Babesia microti and

Plasmodium berghei yoellii infections in nude

mice'. Nature, (London), 252, 328-329.

CLARK, I.A., WILLS, E.J., RICHMOND, Joan E. & ALLISON,

A.C. (1975). 'Immunity to intra-erythrocytic

protozoa'. Lancet, Vol. II of 1975, 1128-1129.

COLAS-BELCOUR, J. & VERVENT, G. (1953). 'Quelques

observations sur des souris ayant survécu à leur

infection par Babesia (Nuttallia) rodhaini'.

Bulletin de la Société de Pathologie Exotique,

46, 34-39.

COLLINS, J.D., O'NUALLÁIN, T.O. & FERGUSON, A.R. (1970).

'Observations on bovine babesiosis in Ireland'.

Irish Veterinary Journal, 24, 42-51.

CORRIER, D.E. & ADAMS, L.G. (1973). 'Observations during

concurrent anaplasmosis and babesiosis in

experimentally infected calves'. Proceedings of

the 6th. National Anaplasmosis Conference,

Las Vegas, Nevada, March, 1973.

COX, F.E.G. (1970). 'Parasitic protozoa of British wild

mammals'. Mammal Review, 1, 1-28.

COX, F.E.G. & TURNER, Sheila A. (1970). 'Antibody levels

in mice infected with Babesia microti'. Annals

of Tropical Medicine and Parasitology, 64, 167-173.



- COX, F.E.G. & YOUNG, A.S. (1969). 'Acquired immunity to Babesia microti and Babesia rodhaini in mice'. Parasitology, 59, 257-268.
- CURNOW, J.A. (1968). 'In vitro agglutination of bovine erythrocytes infected with Babesia argentina'. Nature, (London), 217, 267-268.
- CURNOW, J.A. (1973a). 'The use of a slide-agglutination test to demonstrate antigenic differences between Babesia bigemina parasites'. Australian Veterinary Journal, 49, 290-293.
- CURNOW, J.A. (1973b). 'Studies on antigenic changes and strain differences in Babesia argentina infections'. Australian Veterinary Journal, 49, 279-283.
- CURNOW, J.A. & CURNOW, B.A. (1967). 'An indirect haemagglutination test for the diagnosis of Babesia argentina infection in cattle'. Australian Veterinary Journal, 43, 286-290.
- DACIE, J.V. & LEWIS, S.M. (1966). 'Practical haematology'. 3rd. Ed., J. & A. Churchill Ltd., London.
- DALL, W.H. (1898). 'Synopsis of the recent and Tertiary Psammobiidae of North America'. Proceedings of the Academy of Natural Sciences of Philadelphia, 50, 57-62.
- EBERHART, J.A. (1971). 'Plasma cortisol concentrations in newborn calves'. American Journal of Veterinary Research, 32, 1921-1927.

- DALY, G.D. & HALL, W.T.K. (1955). 'A note on the susceptibility of British and some zebu-type cattle to tick-fever (babesiosis)'. Australian Veterinary Journal, 31, 152.
- DAVIES, S.F.M., JOYNER, L.P. & KENDALL, S.B. (1958). 'Studies on Babesia divergens (M'Fadyean & Stockman, 1911)'. Annals of Tropical Medicine and Parasitology, 52, 206-215.
- DIPEOLU, O.O. (1975). 'The incidence of ticks of Boophilus species on cattle, sheep and goats in Nigeria'. Tropical Animal Health and Production, 7, 35-39.
- DODD, S. (1910). 'Piroplasmosis of cattle in Queensland'. Journal of Comparative Pathology and Therapeutics, 23, 141-160.
- DOLAN, T.T. (1974). 'Mechanisms of pathogenesis in experimental babesiosis'. Ph.D. Thesis, University of Edinburgh.
- DONNELLY, J., JOYNER, L.P. & CROSSMAN, P.J. (1972). 'The incidence of Babesia divergens infection in a herd of cattle as measured by the Indirect Immunofluorescent Antibody Test'. Research in Veterinary Science, 13, 511-514.
- EBERHART, R.J. & PATT, J.A. (1971). 'Plasma cortisol concentrations in newborn calves'. American Journal of Veterinary Research, 32, 1921-1927.

EDWARDS, A.V., HARDY, R.N. & MALINOWSKA, K.W. (1975).

'The sensitivity of adrenal responses to synthetic adrenocorticotrophin in the conscious unrestrained calf'. Journal of Physiology, 245, 639-653.

EWING, S.A. (1965). <sup>'Observations on the persistence and recurrence of</sup> Babesia canis infections in dogs'. Veterinary Medicine, 60, 741-744.

EWING, S.A. (1966). 'Evaluation of methods used to detect Babesia canis infections in dogs'. Cornell Veterinarian, 56, 211-220.

FALLON, G.A. (1965). 'Redwater'. The Veterinary Review (M. & B.), 16, 63-69.

FOLKERS, C. & KUIL, H. (1967). 'Blood parasites in cattle, sheep and goats in Northern Nigeria'. Bulletin of Epizootic Diseases of Africa, 15, 121-123.

FOLKERS, C., KUIL, H. & PERIÉ, N.M. (1967). 'The prevalence of Babesia bovis (Babesia argentina) in the brains of slaughter cattle in Northern Nigeria'. Bulletin of Epizootic Diseases of Africa, 15, 359-361.

FRANÇA, C. (1910). 'Sur la classification des piroplasmes et description de deux formes de ces parasites'. Archivos do Real Instituto Bacteriológico Camara Pestana, 3, 11-18.

FRASER, D., RITCHIE, J.S.D. & FRASER, A.F. (1975). 'The term "stress" in a veterinary context'. British Veterinary Journal, 131, 653-662.

FRIEDHOFF, K.T., ZWART, D. & BROCKLESBY, D.W. (1974).

'Taxonomy and structure of Piroplasmea'.

Proceedings of the Third International Congress of Parasitology, Munich, 1, 11-12.

GAMBLE, P.G. (1974). 'A comparative study of some changes in the blood and cerebral capillaries in mice during infection with Babesia rodhaini and Babesia microti'. M.Sc. Thesis, University of Edinburgh.

GARNHAM, P.C.C. & VOLLER, A. (1965). 'Experimental studies on Babesia divergens in rhesus monkeys, with special reference to its diagnosis by serological methods'. Acta Protozoologica, 3, 183-187.

GARTNER, R.J.W., CALLOW, L.L., GRANZIEN, Corinne K. & PEPPER, Patricia M. (1969). 'Variations in the concentration of blood constituents in relation to the handling of cattle'. Research in Veterinary Science, 10, 7-12.

GATES, D.W. (1953). 'The technique employed in splenectomy of bovine animals'. Journal of the American Veterinary Medical Association, 122, 94-98.

GLEASON, Neva N., HEALY, G.R., WESTERN, K.A., BENSON, G.D. & SCHULTZ, M.G. (1970). 'The 'Gray' strain of Babesia microti from a human case established in laboratory animals'. Journal of Parasitology, 56, 1256-1257.

- GOLDMAN, M. (1968). 'Fluorescent Antibody Methods'.  
1st. Ed., Academic Press, New York and London.
- GOLDMAN, M., PIPANO, E. & ROSENBERG, A.S. (1972).  
'Fluorescent antibody tests for Babesia bigemina  
and B. berbera'. Research in Veterinary Science,  
13, 77-81.
- GOODWIN, L.G. & RICHARDS, W.H.G. (1960).  
'Pharmacologically active peptides in the blood  
and urine of animals infected with Babesia  
rodhaini and other pathogenic organisms'.  
British Journal of Pharmacology and Chemotherapy,  
15, 152-159.
- GRAY, A.R. (1965). 'Antigenic variation in a strain of  
Trypanosoma brucei transmitted by Glossina  
morsitans and G. palpalis'. Journal of General  
Microbiology, 41, 195-214.
- HARVEY, F.T. (1935). 'Some aspects of redwater in  
cattle'. Veterinary Record, 15, 1015-1021.
- HEITZMAN, R.J., ADAMS, T.C. & HUNTER, G.D. (1970).  
'The assay of corticosteroids in plasma of the  
dairy cow'. Journal of Endocrinology, 47,  
133-134.
- HENNING, M.W. (1956). 'Animal Diseases in South Africa'.  
3rd. Ed., Central News Agency Ltd., South Africa.
- HERBERT, W.J. (1975). 'Interference between two strains  
of Trypanosoma brucei'. Transactions of the Royal  
Society of Tropical Medicine and Hygiene, 69, 272.

- HIRSCH, D.C., HICKMAN, R.L., BURKHOLDER, C.R. & SOAVE, O.A. (1969). 'An epizootic of babesiosis in dogs used for medical research'. *Laboratory Animal Care*, 19, 205-208.
- HOFFMAN, G., SCHEIN, E. & MÜLLER, B. (1971). 'Zur Beeinflussung von Babesia bigemina-Parasitämien nach experimentellen Übertragungen mit Boophilus annulatus'. *Berliner und Münchener Tierärztliche Wochenschrift*, 84, 241-246.
- HOOGSTRAAL, H. (1954). 'Noteworthy African tick records in the British Museum (Natural History) Collections'. *Proceedings of the Entomological Society of Washington*, 56, 273-279.
- HOOGSTRAAL, H. (1956). 'African Ixodoidea. I. Ticks of the Sudan'. Department of the Navy Bureau of Medicine and Surgery, Washington D.C.
- HOPWOOD, R.T. & TIBOLLA, B.J. (1958). 'The effect of adrenocorticotrophic hormone on the circulating eosinophil level - a possible screening test for adrenal gland function in the cow'. *American Journal of Veterinary Research*, 19, 833-835.
- HOYTE, H.M.D. (1961). 'Initial development of infection with Babesia bigemina'. *Journal of Protozoology*, 8, 462-466.



- HOYTE, H.M.D. (1971). 'Differential diagnosis of Babesia argentina and Babesia bigemina in cattle using thin blood smears and brain smears'. Australian Veterinary Journal, 47, 248-250.
- IRVIN, A.D. & BROCKLESBY, D.W. (1969). 'Continuous high parasitaemia in mice infected with Babesia (Nuttallia) microti'. Journal of Parasitology, 55, 1190.
- ISIHARA, T. (1968). 'Bovine piroplasmosis in Japan'. Japan Agricultural Research Quarterly, 3, 23-31.
- JOHNSTON, L.A.Y. (1968). 'The incidence of clinical babesiosis in cattle in Queensland'. Australian Veterinary Journal, 44, 265-267.
- JOHNSTON, L.A.Y. & CALLOW, L.L. (1963). 'Intracerebral inoculation and brain biopsy in cattle'. Australian Veterinary Journal, 39, 22-24.
- JOHNSTON, L.A.Y., PEARSON, R.D. & LEATCH, G. (1973). 'Evaluation of an Indirect Fluorescent Antibody test for detecting Babesia argentina infections in cattle'. Australian Veterinary Journal, 49, 373-377.
- JOHNSTON, L.A.Y. & TAMMEMAGI, L. (1969). 'Bovine babesiosis: duration of latent infection and immunity to Babesia argentina'. Australian Veterinary Journal, 45, 445-449.

- JOYNER, L.P. (1966). 'Experimental infections with Babesia divergens'. Journal of Protozoology, 13, Suppl. p. 20.
- JOYNER, L.P. & DAVIES, S.F.M. (1967). 'Acquired resistance to Babesia divergens in experimental calves. Journal of Protozoology, 14, 260-262.
- JOYNER, L.P., DAVIES, S.F.M. & KENDALL, S.B. (1963). 'The experimental transmission of Babesia divergens by Ixodes ricinus'. Experimental Parasitology, 14, 367-373.
- JOYNER, L.P., DONNELLY, J., PAYNE, R. & BROCKLESBY, D.W. (1972). 'The Indirect Fluorescent Antibody Test for the differentiation of infections with Babesia divergens or Babesia major'. Research in Veterinary Science, 13, 515-518.
- JUNGMAN, R. (1966). 'Methods of diagnosing bovine piroplasmosis and their limitations'. [In German]. Angewandte Parasitologie, 7, 196-201.
- KERR, W.R., ROBERTSON, M. & MCGIRR, J.L. (1951). 'A study of the reactions of the white blood corpuscles in bovines at parturition with a consideration of the action of adrenal cortical hormone (cortisone)'. Journal of Hygiene, 49, 67-80.
- LATIF, B.M.A. (1972). 'The immunofluorescent reaction for the diagnosis of protozoal infections in man and animals'. Ph.D. Thesis, University of Edinburgh.

- LATIF, B.M.A. & WELLS, E.A. (1973). 'Babesiasis on the Island of Arran, Scotland'. Veterinary Record, 92, 496-498.
- LEGG, J. (1933). 'A brief review of the piroplasms, with special reference to the types found in Australian "redwater"'. Australian Veterinary Journal, 9, 14-19.
- LEGG, J. (1935). 'The occurrence of bovine babesiellosis in Northern Australia'. Council for Scientific and Industrial Research, Australia, Pamphlet No. 56, pp. 1-48.
- LEEFLANG, P. (1972). 'Diagnosis of Babesia argentina infections in cattle using brain smears'. Australian Veterinary Journal, 48, 72.
- LEEFLANG, P. & PERIÉ, N.M. (1972). 'Comparative immunofluorescent studies on 4 Babesia species of cattle'. Research in Veterinary Science, 13, 342-346.
- LEVINE, N.D. (1961). 'Protozoan Parasites of Domestic Animals and of Man'. Burgess Publishing Co., Minneapolis, Minnesota.
- LEVINE, N.D. (1973). 'Protozoan Parasites of Domestic Animals and of Man'. 2nd. Ed., Burgess Publishing Co., Minneapolis, Minnesota.

LIGNIÈRES, J. (1903). 'La piroplasmose bovine. Nouvelle recherches et observations sur la multiplicité des parasites, leur évolution, la transmission naturelle de la maladie et vaccination'.

Archives de Parasitologie, 7, 398-407.

LÖHR, K.F. (1969). 'Immunisierung gegen Babesiose und Anaplasrose von 40 nach Kenya importierten Charollais-Rindern und Bericht über Erscheinungen der Photosensibilität bei diesen Tieren'.

Zentralblatt für Veterinärmedizin, 16, 40-46.

LÖHR, K.F. (1973). 'Susceptibility of non-splenectomised and splenectomised Sahiwal cattle to experimental Babesia bigemina infection'. Zentralblatt für Veterinärmedizin, 20, 52-56.

LÖHR, K.F. & ROSS, J.P.J. (1969). 'A capillary tube agglutination test for the detection of Babesia bigemina antibodies. [In German]. Zeitschrift für Tropenmedizin und Parasitologie, 20, 287-292.

LUCAS, J.M.S. (1960). 'The chemotherapy of experimental babesiosis in mice and splenectomised calves'. Research in Veterinary Science, 1, 218-225.

LUDFORD, C.G. (1969). 'Fluorescent antibody staining of four Babesia species'. Experimental Parasitology, 24, 327-335.

- LUMSDEN, W.H.R., CUNNINGHAM, M.P., WEBBER, W.A.F.,  
VAN HOEVE, K., KNIGHT, R.H. & SIMMONS, V. (1965).  
'Some effects of hydrogen ion concentration on  
trypanosome numbers and infectivity'.  
Experimental Parasitology, 16, 8-17.
- LUMSDEN, W.H.R. & HARDY, G.J.C. (1965). 'Nomenclature of  
living parasitic material'. Nature, (London),  
205, 1032.
- LYKINS, J.D., RISTIC, M., WEISIGER, R.M. & HUXSOLL, D.L.  
(1975). 'Babesia microti: pathogenesis of  
parasite of human origin in the hamster'.  
Experimental Parasitology, 37, 388-397.
- MACFIE, J.W.S. (1914). 'Notes on some blood parasites  
collected in Nigeria'. Annals of Tropical  
Medicine and Parasitology, 8, 439-468.
- MAEGRAITH, B.G., GILLES, H.M. & DEVAKUL, K. (1957).  
'Pathological processes in Babesia canis  
infections'. Zeitschrift für Tropenmedizin und  
Parasitologie, 8, 485-514.
- MAHONEY, D.F. (1962). 'Bovine babesiosis: diagnosis of  
infection by a complement fixation test'.  
Australian Veterinary Journal, 38, 48-52.
- MAHONEY, D.F. (1964). 'Bovine babesiosis. An assessment  
of the significance of complement fixing antibody  
based upon experimental infection'. Australian  
Veterinary Journal, 40, 369-375.

- MAHONEY, D.F. (1967). 'Bovine babesiosis: preparation and assessment of complement fixing antigens'. *Experimental Parasitology*, 20, 232-241.
- MAHONEY, D.F. (1972). In 'Immunity to Animal Parasites' (Soulsby, E.J.L. ed.), pp. 301-341. Academic Press, New York and London.
- MAHONEY, D.F. & SAAL, J.R. (1961). 'Bovine babesiosis: thick blood films for detection of parasitaemia'. *Australian Veterinary Journal*, 37, 44-47.
- MAHONEY, D.F., WRIGHT, I.G. & MIRRE, G.B. (1973). 'Bovine babesiosis: the persistence of immunity to Babesia argentina and B. bigemina in calves (Bos taurus) after naturally acquired infection'. *Annals of Tropical Medicine and Parasitology*, 67, 197-203.
- MATSON, B.A. (1964). 'Tick transmission in Babesia divergens and immunity in Babesia rodhaini'. Ph.D. Thesis, University of Cambridge, London.
- MATTINGLY, D. (1962). 'A simple fluorimetric method for the estimation of free 11-hydroxycorticoids in human plasma'. *Journal of Clinical Pathology*, 15, 374-379.
- McHARDY, N. (1967). 'Immunisation of rats against Babesia (Nuttallia) rodhaini'. *Nature*, (London), 214, 805.



McHARDY, N. (1974). 'Elimination of Eperythrozoon spp. from mixed infections with Babesia and Anaplasma'. International Journal for Parasitology, 4, 107-108.

McMASTER, P.D. & FRANZL, R.E. (1961). 'The effects of adrenocortical steroids upon antibody formation'. Metabolism, 10, 990-1005.

MEDICAL RESEARCH COUNCIL WORKING PARTY (1971). 'Recommended method for the determination of plasma corticosteroids'. British Medical Journal, 2, 310-313.

MEHLITZ, D. & EHRET, R. (1974). 'Serological investigations on the prevalence of anaplasmosis and piroplasmosis in cattle in Botswana'. Zeitschrift für Tropenmedizin und Parasitologie, 25, 3-10.

MERRILL, W.G. & SMITH, Vearl R. (1954). 'A comparison of some cellular and chemical constituents of blood at time of parturition and after administration of adrenocorticotrophin'. Journal of Dairy Science, 37, 546-551.

M'FADYEAN, J. & STOCKMAN, S. (1911). 'A new species of piroplasm found in the blood of British cattle'. Journal of Comparative Pathology and Therapeutics, 24, 340-354.

- MIESSNER, H. (1931). 'Piroplasmosen und Splenektomie'.  
Archiv für Wissenschaftliche U. Praktische  
Tierheilkunde, 63, 78-90. (Abs. Veterinary  
Bulletin - 1931, 1, 210).
- MOHAMMED, A.N. (1974). 'The seasonal incidence of Ixodid  
ticks of cattle in Northern Nigeria and in the  
Netherlands, with particular reference to their  
role in the transmission of bovine piroplasms'.  
Ph.D. Thesis, University of Zaria.
- NEITZ, W.O. (1938). 'The destruction of Piroplasma canis  
by the neutrophiles and large mononuclear  
leucocytes'. Onderstepoort Journal of Veterinary  
Science and Animal Industry, 10, 33-36.
- NEITZ, W.O. (1941). 'The occurrence of Babesia bovis in  
South Africa'. Journal of the South African  
Veterinary Medical Association, 12, 62-66.
- NEITZ, W.O. (1956). 'Classification, transmission and  
biology of piroplasms of domestic animals'.  
Annals of the New York Academy of Sciences, 64,  
56-111.
- NIGERIA, (1927). Annual Report of the Veterinary  
Department, Northern Province, p. 23. Government  
Printer, Lagos.
- NIGERIA, (1928a). Annual Report of the Veterinary  
Department, Northern Province, p. 27. Government  
Printer, Lagos.
- NIGERIA, (1928b). Ibid., p. 10.

NIGERIA, (1930). Annual Report of the Veterinary  
Department, Northern Province, p. 15. Government  
Printer, Lagos.

NIGERIA, (1934). Annual Report of the Veterinary  
Department, Northern Province, p. 8 et seq.  
Government Printer, Kaduna.

NIGERIA, (1947). Annual Report of the Veterinary  
Department, Northern Province, p. 9. Government  
Printer, Kaduna.

NIGERIA, (1950-51). Annual Report of the Veterinary  
Department, Northern Province, p. 20. Government  
Printer, Kaduna.

NIGERIA, (1963-64). Annual Report of the Veterinary  
Division of the Ministry of Animal Health and  
Forestry, Northern Nigeria, p. 11. Government  
Printer, Kaduna.

NISSLE, A. (1905). 'Blutparasiten und Erythrocytolysen'.  
Archiv für Hygiene und Bakteriologie, 54,  
343-353.

NOWELL, F. (1969). 'The blood picture resulting from  
Nuttallia (=Babesia) rodhaini and Nuttallia  
(=Babesia) microti infections in rats and mice'.  
Parasitology, 59, 991-1004.

OSBORNE, M. (1970). 'Clinical aspects of babesiosis'.  
Irish Veterinary Journal, 24, 52-54.

OVERDULVE, J.P. & ANTONISSE, H.W. (1970). 'Measurement of the effect of low temperature on Protozoa by titration'. *Experimental Parasitology*, 27, 323-341.

PAAPE, M.J., DESJARDINS, C., SCHULTZE, W.D. & SMITH, J.W. (1972). 'Corticosteroid concentrations in jugular and mammary vein blood plasma of cows after over-milking'. *American Journal of Veterinary Research*, 33, 1753-1758.

PAGET, G.E., ALCOCK, S.J. & RYLEY, J.F. (1962). 'Pathology of Babesia rodhaini infections in mice'. *Journal of Pathology and Bacteriology*, 84, 218-220.

PARKER, R. (1973). 'A direct counting technique for estimating high parasitaemias in infections of Babesia argentina, Babesia bigemina and Plasmodium berghei'. *Annals of Tropical Medicine and Parasitology*, 67, 387-390.

PEHRSON, B. & WALLIN, O. (1966). 'A test of adrenal cortical function in dairy cows by A.C.T.H. administration'. *Acta Veterinaria Scandinavica*, Excerptum 7, 1-12.

PEIRCE, M.A. (1975). 'Nuttallia, Franca 1909\* (Babesiidae) pre-occupied by Nuttallia, Dall 1898 (Psammobiidae): a re-appraisal of the taxonomic position of the avian piroplasms'. *International Journal for Parasitology*, 5, 285-287. \* - Sic

- PETERS, W. (1965). 'Competitive relationship between Eperythrozoon coccoides and Plasmodium berghei in the mouse'. *Experimental Parasitology*, 16, 158-166.
- PHILIP, R.G. (1975). 'The preparation of an antigen of Babesia rodhaini for use in the capillary tube agglutination test'. M.Sc. Thesis, University of Edinburgh.
- PHILLIPS, R.S. (1968). 'Studies on Babesia rodhaini in laboratory animals'. Ph.D. Thesis, University of Cambridge, London.
- PHILLIPS, R.S. (1969a). 'Babesia rodhaini infections in the rat after acute primary parasitaemia'. *Parasitology*, 59, 349-356.
- PHILLIPS, R.S. (1969b). 'The role of the spleen in relation to natural and acquired immunity to infections of Babesia rodhaini in the rat'. *Parasitology*, 59, 637-648.
- PIPANO, E., WEISMAN, Y., RAZ, A. & KLINGER, I. (1972). 'Immunity to Babesia bigemina in calves after successful babesicidal treatment of a previous infection'. *Refuah Veterinarith*, 29, 1-8.

- PLOEM, J.S. (1967). 'The use of a vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light'. *Zeitschrift für Wissenschaftliche Mikroskopische*, 68, 129-142.
- PLUMMER, G., HOLLINGSWORTH, D.C., PHUANGSAB, A. & BOWLING, C.P. (1970). 'Chronic infections by Herpes simplex viruses and by the horse and cat Herpes viruses'. *Infection and Immunity*, 1, 351-355.
- POCHE, F. (1913). 'Das System der Protozoa'. *Archiv für Protistenkunde*, 30, 125-310.
- PURCHASE, H.S. (1945). 'A simple and rapid method for demonstrating Rickettsia ruminantium (Cowdry, 1925) in heartwater brains'. *Veterinary Record*, 57, 413-414.
- RANATUNGA, P. & WANDURAGALA, L. (1972). 'Reactions and haematology in imported Jersey cattle premunised in Ceylon'. *British Veterinary Journal*, 128, 9-18.
- RICHARDSON, U.F. & KENDALL, S.B. (1963). 'Veterinary Protozoology'. 3rd. Ed., Oliver and Boyd, Edinburgh and London.
- RIEK, R.F. (1968). In 'Infectious Blood Diseases of Man and Animals' (Weinman, D. & Ristic, M., eds.) Vol. 2, pp. 219-268. Academic Press, New York and London.



RISTIC, M. & SIBINOVIC, S. (1964). 'Equine babesiosis: diagnosis by a precipitation in gel and by a one-step fluorescent antibody-inhibition test'. American Journal of Veterinary Research, 25, 1519-1526.

ROBERTS, J.A. & TRACEY-PATTE, P. (1975a). 'Babesia rodhaini: a study of the effects of immune serum'. International Journal for Parasitology, 5, 577-581.

ROBERTS, J.A. & TRACEY-PATTE, P. (1975b). 'Babesia rodhaini: immuno-induction of antigenic variation'. International Journal for Parasitology, 5, 573-576.

RODHAIN, J. (1950). 'Sur la pluralité des espèces de Babesia des rongeurs. A propos de la spécificité de Babesia rodhaini van den Berghe et al'. Annales de l'Institut Pasteur, Paris, 79, 777-785.

ROGERS, R.J. (1971a). 'An evaluation of tick-fever outbreaks in Northern Queensland in recent years'. Australian Veterinary Journal, 47, 415-417.

ROGERS, R.J. (1971b). 'The acquired resistance to Babesia argentina of cattle exposed to light infestation with cattle tick'. Australian Veterinary Journal, 47, 237-241.

- ROSS, D.R. & MAHONEY, D.F. (1974). 'Bovine babesiasis: computer simulation of Babesia argentina parasite rates in Bos taurus cattle'. Annals of Tropical Medicine and Parasitology, 68, 385-392.
- ROSS, J.P.J. & LÖHR, K.F. (1968). 'Serological diagnosis of Babesia bigemina infection in cattle by the Indirect Fluorescent Antibody Test'. Research in Veterinary Science, 9, 557-562.
- RYLEY, J.F. (1957). 'The chemotherapy of Babesia infections'. Annals of Tropical Medicine and Parasitology, 51, 38-49.
- SCHALM, O.W., JAIN, N.C. & CARROLL, E.J. (1975). 'Veterinary haematology'. 3rd. Ed., Lea and Febiger, Philadelphia.
- SCHERMER, S. (1967). 'The Blood morphology of laboratory animals'. 3rd. Ed., F.A. Davis Co., Philadelphia.
- SCHROEDER, W.F., COX, H.W. & RISTIC, M. (1966). 'Anaemia, parasitaemia, erythrophagocytosis and haemagglutinins in Babesia rodhaini infection'. Annals of Tropical Medicine and Parasitology, 60, 31-38.
- SELYE, H. (1936). 'A syndrome produced by diverse nocuous agents'. Nature, (London), 138, 32.
- SELYE, H. (1956). 'The Stress of Life'. Longmans Green & Co., London.
- SERGEANT, E. (1963). In 'Immunity to Protozoa' (Garnham, P.C.C., Pierce, A.E. & Roitt, I., eds.), pp. 39-47. Blackwell Scientific Publications, Oxford.

SERGEANT, E., PARROT, L. & DONATIEN, A. (1924).

'Une question de terminologie: Immuniser et Prémunir'. Bulletin de la Société de Pathologie Exotique, 17, 37-38.

SERGEANT, E., DONATIEN, A., PARROT, L. & LESTOQUARD, F. (1945). 'Études sur les Piroplasmoses Bovines'. Institut Pasteur d'Algérie.

SERGEANT, E., DONATIEN, A., PARROT, L., LESTOQUARD, F. & PLANTUREUX, E. (1927). 'Études expérimentales sur les piroplasmoses bovines d'Algérie'. Annales de L'Institut Pasteur, Paris, 41, 721-785.

SHORTT, H.E. & BLACKIE, E.J. (1965). 'An account of the genus Babesia as found in certain small mammals in Britain'. Journal of Tropical Medicine and Hygiene, 68, 37-42.

SIBINOVIC, K.H., SIBINOVIC, S., RISTIC, M. & COX, H.W. (1967). 'Immunogenic properties of babesial serum antigens'. Journal of Parasitology, 53, 1121-1129.

SILBER, R.H. & PORTER, C.C. (1957). 'Determination of 17, 21-dihydroxy-20-ketosteroids in urine and plasma'. Methods of Biochemical Analysis, 4, 139-169.

SMITH, T. & KILBORNE, F.E. (1893). 'Investigations into the nature, causation and prevention of Texas or southern cattle fever'. Bulletin. Bureau of Animal Industry, United States Department of Agriculture, 1, 301 pp.

STARCOVICI, C. (1893). 'Bemerkungen über den durch Babes entdeckten Blutparasiten und die durch denselben hervorgebrachten Krankheiten, die seuchenhafte Hämoglobinurie des Rindes (Babes), das Texasfieber (Th. Smith) und der Carceag der Schafe (Babes)'. Zentralblatt für Bakteriologie, Abt. 14, 1-8.

STRICKLAND, K.L. (1961). 'A study of the ticks of the domesticated animals in Northern Nigeria: a preliminary to disease investigation'. M.Sc. Thesis, University of Dublin.

TAKAHASHI, K., YAMASHITA, S., ISAYAMA, Y. & SHIMIZU, Y. (1976). 'Serological response to the Indirect Fluorescent Antibody test of cattle infected with Theileria sergenti'. British Veterinary Journal, 132, 112-117.

TAKAHASHI, K., YAMASHITA, S. & SHIMIZU, Y. (1975). 'Role of antibody detected by the Indirect Fluorescent Antibody test in the clinical signs of cattle infected with Theileria sergenti'. [In Japanese]. Japanese Journal of Veterinary Science, 37, 295-301.

TALIAFERRO, W.H. (1955). In 'Some Physiological Aspects and Consequences of Parasitism' (Cole, W.H., ed.), pp. 50-75. Rutgers University Press, New Brunswick, New Jersey.

- THOONGSUWAN, S. & COX, H.W. (1973). 'Antigenic variants of the haemosporidian parasite, Babesia rodhaini, selected by in vitro treatment with immune globulin'. Annals of Tropical Medicine and Parasitology, 67, 373-385.
- TODOROVIC, R., FERRIS, D. & RISTIC, M. (1967). 'Roles of the spleen in acute plasmodial and babesial infections in rats'. Experimental Parasitology, 21, 354-372.
- TREES, A.J. (1975). 'The application of acridine orange staining to quantitate low levels of Babesia divergens parasitaemias'. Transactions of the Royal Society of Tropical Medicine and Hygiene, 68, 277.
- TSUR, I., HADANI, A. & PIPANO, E. (1960). 'Nuttallia danii n. sp. a haemoprotozoon from the gerbil (Meriones tristrami shawii)'. Refuah Veterinarith, 17, 236-244.
- UDENFRIEND, S. (1969). 'Fluorescence Assay in Biology and Medicine' Vol. II. Academic Press, New York and London.
- UNSWORTH, K. (1952). 'The Ixodid parasites of cattle in Nigeria with particular reference to the Northern Territories'. Annals of Tropical Medicine and Parasitology, 46, 331-336.

- VAN PEENEN, P.F.D. & HEALY, G.R. (1970). 'Infection of Microtus ochrogaster with Piroplasms isolated from man'. *Journal of Parasitology*, 56, 1029-1031.
- V.C.O.U. REPORT (1969). 'Redwater survey 1968'. *Veterinary Record*, 85, 38-39.
- VÖLKER, H., FURCHT, G., STOLPE, J. & BAUER, U. (1973). 'Problems of transport stress in calves under conditions of intensive husbandry. The stress situation and reaction to stress in calves'. [In German]. *Archiv für Experimentelle Veterinärmedizin*, 27, 555-569.
- WATKINS, R.G. (1962). 'A concentration and staining technique for diagnosing equine piroplasmosis'. *Journal of the American Veterinary Medical Association*, 141, 1330-1332.
- WENYON, C.M. (1926). 'Protozoology'. (1965 facsimile of 1926 ed.), Baillière, Tindall and Cassell, London.
- WINTER, H. (1967). 'The diagnosis of babesiosis by fluorescence microscopy'. *Research in Veterinary Science*, 8, 170-174.
- WINTER, H. (1968). 'Direct counting of Protozoa of blood by fluorescence microscopy'. *American Journal of Veterinary Research*, 29, 2419-2422.



- WOLF, R.E. (1974). 'Effects of antilymphocyte serum and splenectomy on resistance to Babesia microti infection in hamsters'. Clinical Immunology and Immunopathology, 21, 381-394.
- WRIGHT, A.I. & WOODFORD, M.H. (1958). 'Bovine piroplasmosis'. Veterinary Record, 70, 627-632.
- WRIGHT, I.G. (1971). 'The isolation of Haemobartonella canis in association with Babesia canis in a splenectomised dog'. Australian Veterinary Journal, 47, 151-159.
- WRIGHT, I.G. (1973). 'Observations on the haematology of experimentally induced Babesia argentina and B. bigemina infections in splenectomised calves'. Research in Veterinary Science, 14, 29-34.
- YOUNG, A.S. (1970). 'Studies on the blood parasites of small mammals with special reference to Piroplasms'. Ph.D. Thesis, University of London.
- YOUNG, A.S. & COX, F.E.G. (1971). 'The effect of B-methasone on Babesia microti and B. rodhaini infections in rodents'. Parasitology, 63, 447-453.
- ZWART, D., PERIÉ, N.M., KEPPLER, A. & GOEDBLOED, E. (1973). 'A comparison of methods for the diagnosis of trypanosomiasis in East African domestic ruminants'. Tropical Animal Health and Production, 5, 79-87.

APPENDICES

Appendix 1

McIlvane's buffer pH 6.4

di-sodium hydrogen phosphate 19.66 gms.

citric acid 6.46 gms.

made up in distilled water to 1 litre and pH 6.4.

Phosphate buffer pH 7.2 - for Giemsa

Prepared using buffer tablets (Hopkins and Williams, Essex).

Phosphate buffer pH 8.0

sodium di-hydrogen phosphate solution (24.02 gms./litre) - 3.6 mls.

di-sodium hydrogen phosphate solution (36.89 gms./litre) - 96.4 mls.

to give 100 mls. at pH 8.0

Phosphate buffered saline pH 7.2

phosphate buffer pH 7.2 (as above) made up with sodium chloride solution (8.5 gms./litre).

Phosphate buffered salt solution (ABP 8.0) - Lumsden, Cunningham, Webber, van Hoeve, Knight & Simmons (1965).

solution 'A' - 9 parts

phosphate buffer pH 8.0 - 1 part

Solution 'A' /.....

Solution 'A'

sodium chloride solution (9.0 gms./litre) -

100 parts

potassium chloride solution (11.48 gms./litre) -

4 parts

magnesium chloride solution (20.94 gms./litre) -

3 parts

calcium chloride solution (22.56 gms./litre) -

1 part

Appendix 2 - Definitions of some terms used

- Absolute parasitaemia - the number of parasitised erythrocytes per unit volume of blood - expressed as a  $\log_{10}$  number and obtained by multiplying the r.b.c. count by the parasitaemia ratio.
- Fever - specifically used to refer to a body temperature in excess of  $39.5^{\circ}\text{C}$ .
- Level of patency - the theoretical minimum parasitaemia which can be detected. It is determined by the number of erythrocytes examined; thus, if  $10^4$  erythrocytes are examined, the level of patency will be 1 in  $10^4$  or 0.01%.
- Minimum relapse interval - the time between consecutive peaks of recrudescent parasitaemias whether these be patent or not.
- Onset of latency - the time when parasitaemia becomes sub-patent (this will be affected by the 'level of patency' adopted).
- Post-infection - as in 'days post-infection' - days since inoculation with parasites.
- Primary parasitaemia - the first parasitaemic episode suffered by a susceptible animal when it is infected. Usually terminated by the 'onset of latency'.
- Relapse interval - the time between consecutive observed peaks of recrudescent parasitaemias. If the parasitaemia is continuously patent this will be the same as the 'minimum relapse interval'.